Exhibit A

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ORIGINAL RESEARCH ARTICLE

Evaluation of linkage of bipolar affective disorder to chromosome 18 in a sample of 57 German families

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Previously reported linkage of bipolar affective disorder to DNA markers on chromosome 18 was reexamined in a large sample of German bipolar families. Twenty-three short tandem repeat markers were investigated in 57 families containing 103 individuals with bipolar I disorder (BPI), 26 with bipolar II disorder (BPII), nine with schizoaffective disorder of the bipolar type (SA/BP), and 38 individuals with recurrent unipolar depression (UPR). Evidence for linkage was tested with parametric and non-parametric methods under two definitions of the affected phenotype. Analysis of all 57 families revealed no robust evidence for linkage. Following previous reports we performed separate analyses after subdividing the families with respect to the sex of the transmitting parent. Fourteen families were classified as paternal and 12 families as maternal. In 31 families the parental lineage of transmission of the disease could not be determined ('either' families). Evidence for linkage was obtained for chromosomal region 18p11.2 in the paternal families and for 18q22–23 in the 'either' families. The findings on 18p11.2 and 18q22–23 support prior evidence for susceptibility loci in these regions. The parent-of-origin effect on 18p11.2 is confirmed in our sample. The delineation of characteristics of 'either' families requires further study.

Although the etiology of bipolar affective disorder is unknown, strong support for an important genetic component comes from the results of family, twin, and adoption studies.[1] Linkage studies of bipolar disorder to date have provided suggestive evidence in favor of locus heterogeneity. Promising chromosomal regions suggested by recent linkage studies include regions on chromosome 18.

Berrettini *et al*[2] first reported linkage of bipolar disorder to a region near the centromere on chromosome 18p in 22 families using the affected-sib-pair (ASP) method and the affected-pedigree-member (APM) method. Parametric LOD score analysis of all 22 families revealed negative LOD scores. However, individual families yielded LOD scores >1 assuming dominant or recessive genetic models. Confirmatory evidence for a bipolar susceptibility locus in this chromosomal region was found by Stine *et al*.[3] Both parametric LOD score analysis and ASP analysis supported linkage in their study of 28 families. In addition, the same study reported a second susceptibility locus on the long arm of chromosome 18 (18q21). Interestingly, linkage to loci on both 18p and 18q was strongest in those families, in which the father or one of the father's siblings was affected, suggesting a parent-of-origin effect operating in bipolar disorder. Gershon *et al*[4] re-analyzed the 18p marker data of Berrettini *et al*[2] by the sex of the transmitting parent. Although no kindred with limited paternal transmission was observed, ASP analysis yielded highly significant excess allele sharing in the pedigrees with mixed maternal-paternal transmission (in different pedigree branches) but not in pedigrees with exclusively maternal transmission confirming the observation of Stine *et al*.[3] Further evidence for a bipolar susceptibility locus on the long arm of chromosome 18 was provided by four recent linkage studies. Freimer *et al*[5] analyzed two large Costa Rican families. Linkage disequilibrium based methods suggested a locus in 18q22–23, whereas the LOD score analysis provided only weak support for the presence of linkage. De bruyn *et al*[6] found linkage of bipolar disorder to markers in 18q21.33–q23 in a large Belgian family using both LOD score and ASP analyses. Coon *et al*[7] obtained weak evidence for linkage at 18q23 in a sample of 13 families from Utah. LOD score analyses revealed positive LOD scores for three markers in this region. No positive results were obtained with APM and ASP analyses. Using LOD score analysis, Ewald *et al*[8] reported linkage of markers to a more proximal part on the long arm of chromosome 18 (q12) in two Danish families. In a study of a 30-family sample, McMahon *et al*[9] obtained confirmatory evidence for a locus at 18q21 using ASP analysis. A parent-of-origin effect was observed, but it was not consistently paternal. No strong evidence for a potential locus on chromosome 18 was found in the recent collaborative NIMH study on bipolar affective disorder.[10] Only one marker at chromosome 18p11.2 and one marker at 18q23 yielded a slight elevation in allele sharing. It is clear from simulations of additive oligogenic inheritance that expectations of universal agreement regarding reported linkages to bipolar disorder are unrealistic.[11] Suarez *et al*[11]

have shown that a much smaller family sample may be sufficient to initially detect linkage to one of the trait loci than to replicate linkage to that locus. Therefore, it might not be unexpected that some other studies have not been able to support linkage with chromosome 18.[12–16] On the other hand, linkage results must be replicated to be credible. If results from two or more independent studies provide significant evidence for linkage in independent series of pedigrees, it is reasonable to assume validity.[17] In the current study we wanted to test the validity of the recent linkage reports investigating a large series of 57 small to moderate German pedigrees. We analyzed 23 microsatellite markers from chromosome 18. The majority of markers were localized on 18p11.2 and 18q21.2–q23; the regions on chromosome 18 being most consistently implicated by previous studies.

LOD score analysis

Twenty-three markers were genotyped on chromosome 18. The LOD scores are shown in Table 1. Overall two-point LOD scores did not exceed 1.0 for either model, except for marker D18S37 (1.08 at $\theta = 0.1$) under a recessive genetic model and ASM II. When we divided the pedigrees into maternal, paternal, and 'either' families, LOD scores >1 were seen in the paternal families for markers D18S453 (1.91 at $\theta = 0.0$), D18S37 (1.67 at $\theta = 0.0$), and D18S40 (1.65 at $\theta = 0.01$) under a recessive genetic model and ASM I, and for D18S53 (1.06 at $\theta = 0.0$) under a dominant genetic model and ASM I. Under a recessive model and ASM II, marker D18S453 yielded a LOD score of 1.34 at $\theta = 0.0$. In the maternal families LOD scores were below 1.0 for all models. In the 'either' families LOD scores >1 were obtained for D18S469 (1.17 at $\theta = 0.0$) and D18S70 (1.12 at $\theta = 0.05$) under a dominant model and ASM II, for D18S554 (1.59 at $\theta = 0.05$) and D18S70 (1.65 at $\theta = 0.05$) under a dominant model and ASM I, and for D18S554 (1.26 at $\theta = 0.1$) and D18S70 (1.09 at $\theta = 0.1$) with a recessive model and ASM I.

Parametric multipoint LOD score analyses showed peaks in the same regions where two-point LOD scores >1 had been observed (Figure 1): a LOD score of 2.54 was observed in the paternal families at markers D18S37, D18S453, and D18S40 using a recessive genetic model and a stringent phenotype definition. The non-parametric Z-all score in this region peaked at 1.34 ($P = 0.03$) (Figure 1). For the 'either' families, we obtained a maximum parametric LOD score of 2.1 at markers D18S554 and D18S461 under a dominant model and ASM I. The Z-all score was 2.20 ($P = 0.0006$) (Figure 1).

Affected-sib-pair analysis

ASP analyses in all families showed excess allele sharing for D18S40 ($P = 0.035$) under ASM II and a non-significant trend for flanking markers. In the paternal and maternal families, we observed excess allele sharing for the following markers: D18S453 ($P = 0.054$) under ASM II in the paternal pedigrees and D18S36 ($P = 0.0064$) under ASM II in the maternal pedigrees. In the 'either' families, we found excess allele sharing for D18S71 under ASM II. Using ASM I excess allele sharing was observed for D18S554 ($P = 0.017$) and D18S70 ($P = 0.032$).

Many reports of linkage in psychiatric disorders have been published in recent years. Interpretation of these findings is difficult and requires critical consideration of the study design, eg power of the family sample, informativity of markers, and statistical methods. The application of statistical thresholds[17] as guidelines for the interpretation of linkage findings alone may not be sufficient to reflect the complex situation in multifactorial disease.[18,19] Therefore, until susceptibility genes are identified, replication of linkage findings will be the most important criterion to assess their validity.

Although in our study LOD scores failed to reach 3, our results do seem to provide some support for the existence of susceptibility loci on both the short and long arm of chromosome 18. Interestingly, positive findings were obtained after subdividing the families according to the gender of the disease-transmitting parent. Our linkage data support the modest evidence for linkage with markers on 18p11.2 originally reported by Berrettini *et al*.[2,20] In accordance with the results of Stine *et al*[3] and Gershon *et al*[4] we found the highest LOD scores in the subset of paternal families. Moreover, our multipoint LOD score peaked at the same loci (D18S37, D18S453, D18S40; Figure 1) where Gershon *et al*[4] and Stine *et al*[3] found their highest LOD scores on 18p11.2. Thus, this is the third study with a large family sample to find evidence for a susceptibility locus for bipolar disorder in a relatively narrow region on the short arm of chromosome 18. Interestingly, in the collaborative NIMH study the marker D18S40 was the only marker on 18p which showed significantly elevated allele sharing.[10] Unfortunately, no data have yet been published from this sample where the sample was subdivided according to the sex of the transmitting parent.

A paternal effect operating in bipolar disorder is also supported by clinical data.[21–23] Grigoroiu-Serbanescu *et al*[21] found that paternal transmission of the disease was associated with a significantly younger age of onset of bipolar illness in probands. McMahon *et al*[23] observed an increased rate of major affective disorder among maternal relatives and a higher than expected proportion of families with no paternal transmission.

A possible biological explanation for the paternal effect observed in the linkage studies is that a susceptibility gene on 18p underlies genomic imprinting. An alternative explanation might be differences between male and female recombination rates on chromosome 18.[3,24,25] Given a simple fully penetrant dominant or recessive mode of inheritance, it is clear that different allele sharing should occur in paternal and maternal families, simply if the male and female recombination rates between disease and marker locus are different. If, for instance, the male recombination rate is higher than the female recombination rate, one would expect higher sharing in the maternal families for a dominant

Table 1 Results of parametric LOD score analyses

Marker	Genetic map		Dominant broad				Dominant narrow			
	Female	Male	All pedigrees (n = 57) Lod (theta max.)	Paternal (n = 14) Lod (theta max.)	Maternal (n = 12) Lod (theta max.)	Either (n = 31) Lod (theta max.)	All pedigrees (n = 57) Lod (theta max.)	Paternal (n = 14) Lod (theta max.)	Maternal (n = 12) Lod (theta max.)	Either (n = 31) Lod (theta max.)
PACAP	0.000	0.000	–	0.02 (0.4)	–	–	–	–	–	–
D18 S 62	0.104	0.5	–	–	0.06 (0.4)	–	–	–	–	0.03 (0.3)
D18 S 53	0.5	0.135	0.32 (0.2)	–	–	0.23 (0.2)	0.05 (0.3)	1.06 (0.0)	–	–
GOLF	0.01	0.001	0.35 (0.2)	–	0.66 (0.1)	0.43 (0.1)	0.08 (0.3)	0.09 (0.2)	0.14 (0.2)	–
D18 S 71	0.014	0.02	0.17 (0.3)	0.21 (0.01)	0.10 (0.2)	0.49 (0.05)	0.07 (0.3)	0.36 (0.1)	0.01 (0.3)	0.06 (0.3)
D18 S 37	0.001	0.021	0.62 (0.1)	0.16 (0.1)	0.10 (0.2)	0.60 (0.05)	0.02 (0.3)	0.57 (0.1)	0.18 (0.0)	–
D18 S 453	0.001	0.001	0.49 (0.2)	0.30 (0.1)	0.03 (0.3)	0.37 (0.2)	–	0.47 (0.1)	–	–
D18 S 40	0.025	0.008	0.44 (0.2)	–	0.33 (0.05)	0.48 (0.1)	–	0.39 (0.2)	–	–
D18 S 44	0.030	0.015	0.15 (0.3)	–	0.43 (0.2)	0.01 (0.4)	–	0.07 (0.2)	–	–
D18 S 36	0.141	0.028	0.49 (0.2)	–	0.78 (0.1)	0.15 (0.2)	–	0.01 (0.3)	0.30 (0.1)	–
D18 S 39	0.702	0.211	0.04 (0.3)	–	–	0.37 (0.2)	–	–	–	0.09 (0.3)
D18 S 41	0.029	0.008	0.26 (0.2)	0.01 (0.3)	0.03 (0.3)	0.42 (0.1)	–	–	–	0.10 (0.3)
D18 S 64	0.054	0.023	–	–	–	0.17 (0.2)	–	–	–	–
D18 S 38	0.011	0.015	–	–	–	0.12 (0.2)	–	–	–	0.27 (0.2)
D18 S 1147	0.012	0.034	–	–	–	0.65 (0.1)	0.16 (0.3)	–	–	1.06 (0.1)
D18 S 68	0.014	0.058	–	–	–	0.25 (0.2)	–	–	–	0.54 (0.1)
D18 S 392	0.001	0.001	–	–	–	0.45 (0.2)	–	–	–	0.32 (0.2)
D18 S 541	0.125	0.87	0.01 (0.4)	–	–	0.78 (0.1)	–	–	–	0.12 (0.3)
MBP	0.132	0.090	–	–	–	0.55 (0.1)	–	–	–	0.90 (0.05)
D18 S 469	0.119	0.048	0.02 (0.3)	0.01 (0.3)	–	1.17 (0.0)	–	–	–	0.75 (0.1)
18 S 554	0.161	0.064	–	–	–	0.81 (0.1)	0.18 (0.3)	0.06 (0.3)	–	1.59 (0.05)
D18 S 461	0.010	0.082	0.43 (0.2)	0.06 (0.2)	–	0.67 (0.1)	0.25 (0.2)	–	–	0.85 (0.05)
D18 S 70	0.014	0.109	0.31 (0.2)	0.11 (0.2)	–	1.12 (0.05)	0.78 (0.2)	0.11 (0.3)	–	1.65 (0.05)

Continued

Table 1 Continued

Marker	Genetic map		Recessive broad				Recessive narrow			
	Female	Male	All pedigrees (n = 57) Lod (theta max.)	Paternal (n = 14) Lod (theta max.)	Maternal (n = 12) Lod (theta max.)	Either (n = 31) Lod (theta max.)	All pedigrees (n = 57) Lod (theta max.)	Paternal (n = 14) Lod (theta max.)	Maternal (n = 12) Lod (theta max.)	Either (n = 31) Lod (theta max.)
PCAP	0.000	0.000	–	–	0.14 (0.2)	–	0.01 (0.4)	–	0.38 (0.1)	–
D18 S 62	0.104	0.5	–	–	–	0.05 (0.3)	–	–	–	–
D18 S 53	0.5	0.135	0.17 (0.2)	0.30 (0.05)	–	0.05 (0.3)	–	0.96 (0.05)	0.01 (0.4)	–
GOLF	0.01	0.001	0.48 (0.1)	0.05 (0.2)	0.03 (0.2)	0.14 (0.2)	0.28 (0.2)	0.78 (0.05)	0.08 (0.2)	–
D18 S 71	0.014	0.02	0.76 (0.1)	0.83 (0.0)	–	0.99 (0.0)	0.18 (0.3)	0.80 (0.05)	0.06 (0.3)	0.01 (0.4)
D18 S 37	0.001	0.021	**1.08 (0.1)**	0.25 (0.1)	0.11 (0.1)	0.50 (0.1)	0.32 (0.2)	**1.67 (0.0)**	0.32 (0.05)	–
D18 S 453	0.001	0.001	0.75 (0.1)	**1.34 (0.0)**	0.01 (0.4)	0.27 (0.2)	0.30 (0.3)	**1.91 (0.0)**	0.16 (0.2)	–
D18 S 40	0.025	0.008	–	0.22 (0)	0.56 (0.01)	0.14 (0.2)	0.40 (0.2)	**1.65 (0.01)**	0.39 (0.05)	–
D18 S 44	0.030	0.015	0.16 (0.3)	–	0.01 (0.4)	–	–	0.89 (0.05)	–	–
D18 S 36	0.141	0.028	0.10 (0.3)	–	0.32 (0.1)	0.17 (0.2)	–	0.09 (0.2)	0.07 (0.2)	–
D18 S 39	0.702	0.211	0.50 (0.2)	–	0.02 (0.3)	0.13 (0.2)	0.24 (0.3)	0.15 (0.2)	–	0.19 (0.2)
D18 S 41	0.029	0.008	–	–	0.21 (0.1)	0.25 (0.2)	0.33 (0.2)	0.02 (0.3)	0.44 (0.01)	0.12 (0.3)
D18 S 64	0.054	0.023	0.01 (0.4)	–	–	0.12 (0.2)	–	0.02 (0.3)	–	–
D18 S 38	0.011	0.015	0.12 (0.3)	–	–	0.13 (0.2)	–	–	–	0.22 (0.2)
D18 S 1147	0.012	0.034	0.12 (0.3)	–	–	0.28 (0.2)	0.27 (0.3)	0.02 (0.3)	–	0.68 (0.2)
D18 S 68	0.014	0.058	0.01 (0.4)	–	–	0.36 (0.2)	0.07 (0.3)	–	–	0.62 (0.2)
D18 S 392	0.001	0.001	–	–	–	0.43 (0.2)	0.01 (0.4)	–	–	0.38 (0.2)
D18 S 541	0.125	0.87	–	–	–	0.68 (0.1)	–	–	–	0.10 (0.3)
MBP	0.132	0.090	–	–	–	0.46 (0.2)	0.19 (0.3)	–	–	0.87 (0.1)
D18 S 469	0.119	0.048	0.13 (0.3)	–	–	0.82 (0.05)	0.25 (0.2)	–	–	0.98 (0.1)
D18 S 554	0.161	0.064	0.46 (0.2)	–	0.20 (0.1)	0.84 (0.1)	0.47 (0.2)	–	–	**1.26 (0.1)**
D18 S 461	0.010	0.082	0.45 (0.2)	–	0.30 (0.1)	0.42 (0.1)	0.05 (0.3)	–	–	0.60 (0.2)
D18 S 70	0.014	0.109	0.31 (0.2)	–	0.07 (0.2)	0.91 (0.1)	0.82 (0.2)	0.15 (0.2)	–	**1.09 (0.1)**

Note: LOD scores are given for two disease models (broad and narrow) and two genetic models (dominant and recessive). Results are shown for all pedigrees and separate analyses of 'paternal', 'maternal', and 'either' families. Only positive LOD scores are reported (theta max. < 0.5). The genetic map was calculated by means of the LINKMAP program of the LINKAGE package.[36]

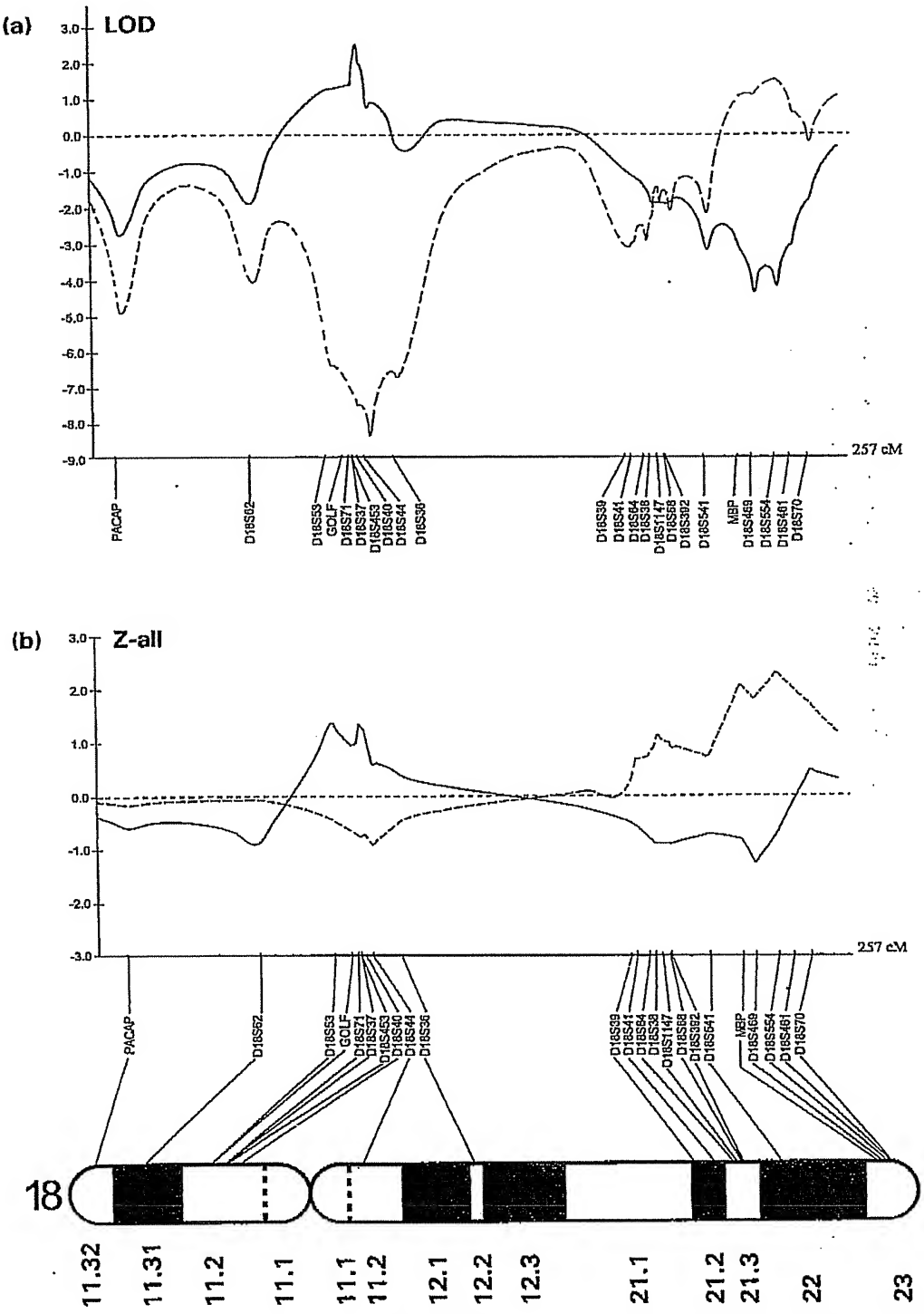

Figure 1 (a) Parametric and (b) non-parametric multipoint linkage analyses of chromosome 18 markers in paternal (straight line) and 'either' (dashed line) families.

model and higher sharing in the paternal families for a recessive model. Under a dominant model, information about linkage between disease and marker locus mainly comes from the transmission of marker alleles from the affected parent to the affected sibs. That means that the information about linkage is in the transmission from father to affected sib in paternal families and in the transmission from mother to the affected children in the maternal families. If we now assume the male recombination rate to be higher, more evidence for linkage is expected to come from the maternal families since here the recombination rate for the relevant transmissions is lower. If a recessive mode of inheritance is assumed, information is mainly contributed by the non-affected parent (ie, from the paternal side in the maternal families and vice versa). Consequently, in this situation a higher male recombination rate will reduce the sharing rate in the maternal families.

To investigate the potential effect of different recombination rates we performed a simulation study. Based on the genetic models used in our analyses we generated 1000 simulations of 100 extended sib pair families. In addition to the marker and disease information of the affected sibs and their parents we assumed to have disease information on the grandparents. This imitates the real situation where in the case when both parents are unaffected the disease status of grandparents is used to assign a family to the paternal or maternal group, respectively. In our simulations we assumed a male recombination rate of 5% and a female recombination rate of 20%. Table 2 shows the allele sharing observed in the different types of families (paternal, maternal, and either families). As expected, allele sharing was higher in paternal families for the dominant models and higher in maternal families for the recessive models. However, when we looked at the power to detect a significant deviation from random allele sharing we found that the power was always higher in the combined data set than in the different subsamples (Table 2). In addition to the evidence given by the simulation study there are other arguments that the positive findings in paternal pedigrees are not simply due to sex differences in recombination. If the findings were caused by this mechanism, one would expect a plethora of falsely positive linkage reports in this region of 18p and not expect an inflation in the number of negative linkage reports, because such linkage studies would presumably detect the same increased non-specific allele sharing. But several such negative reports have appeared.[12–16] Also, when the male and female recombination rates are compared across different regions of chromosome 18, the largest discrepancies occur on 18q.[24,25] Discrepancies on 18p near the centromere are much smaller. Our linkage analyses of markers from the long arm of chromosome 18 provide some support for a second susceptibility locus for bipolar disorder on chromosome 18. The chromosomal region 18q22–23 has originally been proposed by Freimer *et al*[5] who studied two large pedigrees from a genetically homogeneous population in Costa Rica. Their systematic genome screen revealed three markers on chromosome 18 (D18S64, D18S61, and D18S1161) that exceeded a LOD score threshold of 1.6. Fine mapping in this region with additional markers using association analyses produced significant *P*-values for D18S469, D18S554, D18S461, and D18S70. Except for D18S461, these markers yield LOD scores >1.0 in the 'either' subgroup of our family sample as well. Allele sharing IBD was >50% for all four markers with significant *P*-values for D18S554 and D18S70.

Surprisingly, evidence for linkage to 18q22–23 is restricted to the 'either' subgroup of our families. A comparison of results between paternal and 'either' families reveals opposite findings for these two subgroups. While the paternal families are linked to 18p11.2 and display exclusion of linkage to 18q22–23, we observe exclusion of linkage to 18p11.2 in the 'either' families and evidence for linkage to 18q22–23. A possible interpretation of these results is that two different genetic transmission patterns are separated to a considerable extent by the division of pedigrees into maternal, paternal, and 'either' families. Initially, we simply considered the 'either' families a sample which could not be classified into maternal or paternal families. However, if the 'either' families were just a mixture of paternal and maternal families that cannot be separated because of limited pedigree size we would actually not expect the observed positive linkage finding on 18q22–23. Neither paternal nor maternal families give evidence for linkage in this area which makes it unlikely that a mixture of both produces a positive result. Comparison of the family structures between the

Table 2 Simulation of effects of sex differences in recombination rates

Genetic model	*Allele sharing*				*Power*			
	Paternal families	*Maternal families*	*Either families*	*All families*	*Paternal families*	*Maternal families*	*Either families*	*All families*
Dominant broad	64% ± 7%	57% ± 7%	58% ± 6%	59% ± 5%	71%	30%	40%	83%
Dominant narrow	69% ± 5%	59% ± 6%	64% ± 7%	64% ± 3%	96%	45%	66%	99%
Recessive broad	60% ± 9%	63% ± 8%	61% ± 5%	61% ± 4%	39%	53%	77%	93%
Recessive narrow	63% ± 10%	70% ± 9%	75% ± 4%	72% ± 3%	50%	75%	100%	100%

subgroups shows that the parent's affection status is a prominent difference: in contrast to maternal or paternal families where in most cases one parent is affected, the main proportion of the 'either' subgroup (27 out of 31 families) represents families where none of the probands' parents has a psychiatric diagnosis that is included in our disease models. This prominent family structure in the subgroup of 'either' families may reflect a recessive gene action. Surprisingly, we found the most significant results using a dominant inheritance model. Further studies of independent family samples may help to clarify this issue. Of course, there may be other, not yet recognized characteristics that distinguish between 'either' and maternal/paternal families.

In accordance with Freimer *et al*,[5] De bruyn *et al*,[6] and Coon *et al*[7] our results support a bipolar locus at 18q22–23. Other linkage results on the long arm of chromosome 18 have been reported by Stine *et al*[3] and McMahon *et al*[9] on q21 and Ewald *et al*[8] on q12. Similar to our study, Stine *et al*[3] reported evidence for two bipolar susceptibility loci on chromosome 18. The exact location of the putative disease locus on 18q may be difficult to determine due to genetic heterogeneity and the probably low relative risk conferred by this locus. Therefore, the possibility cannot be ruled out that all linkage studies on 18q detect the same disease locus. Indeed, subsequent ASP analyses of the Costa Rican kindreds[26] revealed nominally significant allele sharing at D18S64, one of the most significant markers in the report by Stine *et al*.[3]

We note that our linkage findings on chromosome 18 should be interpreted in the light of statistical testing on the two genetic models and the two affected status models. Likewise, the same data are scrutinized using several analytical methods. Clearly, multiple testing has an inflationary impact on *P* levels. However, we believe that there is no clear way of correcting for these effects, which are common to many linkage analyses of complex traits.

Any single study will be insufficient to provide convincing proof for a susceptibility locus in a complex disease because of unknown mode of inheritance, genetic heterogeneity, and nongenetic factors. Although our results do not allow definitive conclusions, they are consistent with the hypothesis that susceptibility loci are present on the short and long arm of chromosome 18. It might well be that for a complex disease such as manic depression it would be unrealistic to expect to obtain much stronger evidence from a data set of this size and that definitive conclusions will need to be based on consideration of results from many independent sources.

Material and methods

Family ascertainment

Families were recruited in six clinical centers (Wurzburg, Mainz, Haar, Dresden, Munchen, Bonn) in Germany. Standard diagnostic definitions for ascertainment and extension are the following: bipolar I (BPI), schizoaffective, bipolar type (SA/BP), unipolar, recurrent (UPR), unipolar, single episode (UPS) and other minor psychiatric disorders are defined by DSMIII-R criteria;[27] bipolar II (BPII) by Research Diagnostic Criteria (RDC)[28] with the modification that it requires recurrent episodes of depression. The diagnosis of BPII cannot be made in DSMIII-R, but is made in RDC. We additionally specified that depression be recurrent because of concerns about the reliability of hypomania and single-episode major depression.[29]

Inclusion criteria for the systematically ascertained BP families were: (1) a proband with BPI and admission to one of the treatment facilities screened; (2) a secondary affected sib with either BPI, BPII, SA/BP, or UPR; and (3) availability of both parents or—if only one parent was accessible—availability of at least two more sibs from the sibship of the proband.

All individuals were interviewed by an experienced psychiatrist using the Schedule for Affective Disorders and Schizophrenia—Lifetime Version (SADS-L).[30] Best estimate diagnoses were based on the interview, review of all available clinical records and family history information.

Description of families

The family sample consisted of 385 individuals from 57 families. None of these families had previously been included in a linkage study with markers from chromosome 18. Results from a subsample of the present sample have previously been published for markers on chromosome 12.[31] The mean number of individuals per pedigree was 6.8. The distribution of diagnoses was as follows: 103 individuals with BPI, 26 individuals with BPII, nine individuals with SA/BP, 38 individuals with UPR, and 38 individuals with a minor psychiatric diagnosis. One hundred and seventy-one individuals were unaffected.

Except for five families (WUE49, WUE67, WUE78, MAI18, HAA117), there were no families where both parents of the index case had a psychiatric diagnosis. In family WUE49, the father had a diagnosis of BPI, and the mother had a diagnosis of UPR. The father of family WUE78 displayed an adjustment disorder with depressed mood, the mother had UPS. The father of family WUE67 had UPR, the mother was BPI. In family MAI18 the father was diagnosed with UPS, the mother with BPII. Both father and mother of family HAA117 had a personality disorder. There were 14 'paternal families', in which the father of the proband, the grandfather or grandmother on the paternal side or at least one of the father's sibs was affected with BPI, BPII, SA/BP, or UPR, respectively. Similarly, we classified 12 families as 'maternal'. For the remaining 31 families, the parental lineage of transmission of the disease could not be determined. These families were designated 'either' families. Twenty-seven of these families could not be classified, because none of the parents was affected and there were no parental relatives available or there were no affected relatives in either parental lineage. In two of the families with two or more branches (MA121, DRE19), we observed both maternal

and paternal transmission of the disease and therefore classified them 'either'. The four families with both parents of the index case having a psychiatric diagnosis were classified as follows: families WUE49, WUE78, and HAA117 were treated as 'either' families, MAI18 was a maternal family. The family classifications were made blind to linkage results.

The 14 paternal families included 102 individuals: 29 BPI, nine BPII, one SA/BP, 12 UPR, four with a minor psychiatric diagnosis and 47 unaffected. In the 12 maternal families, there were 98 individuals: 24 BPI, 10 BPII, one SA/BP, 14 UPR, nine with a minor psychiatric diagnosis and 40 unaffected. The 31 'either' families consisted of 185 individuals: 50 BPI, seven BPII, seven SA/BP, 12 UPR, 25 with a minor psychiatric diagnosis and 84 unaffected.

DNA isolation and cell lines

EDTA anticoagulated venous blood samples were collected from 320 individuals who were available for the study. Leukocyte DNA was isolated as described.[32] Whenever possible, leukocytes were isolated and transformed using Epstein–Barr virus in order to establish permanent cell lines.

Genotyping

Complete genotypic data were collected from 320 individuals. We studied 23 short tandem repeat (STR) markers on chromosome 18 (PACAP, D18S62, D18S53, GOLF, D18S71, D18S37, D18S453, D18S40, D18S44, D18S36, D18S39, D18S41, D18S64, D18S38, D18S1147, D18S68, D18S392, D18S541, MBP, D18S469, D18S554, D18S461, D18S70). Markers D18S64, D18S68, D18S469, and D18S70 were taken from the ABI Prism Linkage Mapping Set (Applied Biosystems, Foster City, CA, USA). Information on primer sequences for all other markers was taken from published marker maps[33–35] (Genome Data Base Version 5.6).

One oligonucleotide of each pair of PCR primers was fluorescein labeled. The PCR reaction for each marker was carried out in a 10-μl volume containing 40 ng genomic DNA, 5 pmol of each primer, 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5–2.5 mM $MgCl_2$, and 0.6 U Taq polymerase (Life Technologies, Rockville, MD, USA). After an initial denaturation of 5 min at 95°C, 33 cycles of amplification of 15 s at 94°C, 15 s at 55–62°C, and 30 s at 72°C were performed in a Perkin Elmer 9600 thermocycler. The resulting amplified products were separated on 4% denaturing polyacrylamide gels on an automated DNA sequencer (Model 377, Applied Biosystems). Allele sizes were determined relative to an internal size standard in each lane using Genescan Analysis and Genotyper software (Applied Biosystems). In addition, a reference individual with known genotype was loaded on each gel. All gels were scored independently by two individuals who were blind to the disease status. Each marker in every family was tested for Mendelian inheritance.

Linkage analyses

Two models of affection were used in the linkage analyses: affection status model (ASM)-I (narrow definition of the phenotype) included only individuals with BPI as affected, all other psychiatric diagnoses were coded as 'unknown'; ASM II (broad definition) included all individuals with a diagnosis of BPI, BPII, SA/BP, and UPR with UPS and other minor psychiatric disorders coded 'unknown'.

There are about 115 ASPs in our 57 pedigrees. This number of ASPs has a power greater than 90% to detect linkage ($\theta \leq 0.05$) at a level $\alpha = 5\%$ under ASM II (recessive and dominant) if at least 50% of the kindreds are linked to a single locus.

Two-point LOD scores were calculated by means of MLINK and ILINK programs of the LINKAGE package.[36] All analyses were conducted using an 'affecteds-only' approach. For both disease definitions the LOD scores were calculated assuming both a dominant and a recessive mode of inheritance. Under the broad affection model we assumed a phenocopy rate of 3.2% and a penetrance of 50% under both genetic models. The frequency of the disease allele was set to 24.5% and 3% for the recessive and dominant genetic model, respectively. For the narrow affection model the phenocopy rate was set to 0.1%, penetrance was assumed to be 50% and the frequency of the disease allele was set to 13.4% under recessive and to 0.9% under dominant mode of inheritance. These assumptions correspond to a lifetime morbid risk of 6% for diseases included in ASM II and of 1% for BPI (ASM I).[37] An age-dependency of the penetrance was not taken into account, since using 'affecteds-only' treats unaffected individuals as phenotype unknown, which diminishes the effect of quantifying the correct penetrance.

The *P*-values for allele sharing between affected sibs were derived by comparing the observed sharing rate for a marker with the exact distribution of allele sharing expected under the null hypothesis of no linkage given the number and size of sib-ships which were informative of the marker. The (one-sided) *P*-value was taken as the probability to have the observed or a higher sharing rate under the null hypothesis.

Multipoint LOD scores as well as nonparametric multipoint analyses were performed using the GENEHUNTER program Version 1.1.[38] For the multipoint LOD score analysis, the same models as for the two-point analysis were used (dominant and recessive mode of inheritance, narrow and broad disease model). For non-parametric multipoint linkage analysis both pairwise identical by descent (IBD), allele sharing (Z-pairs) and IBD sharing among all affected family members (Z-all) was calculated. Only results from the Z-all statistics are given because testing whether the same allele is found IBD in many affected relatives is a more powerful strategy than considering one relative pair at a time.[38]

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Exhibit B

ORIGINAL ARTICLE

Linkage of Bipolar Disorder to Chromosome 18q and the Validity of Bipolar II Disorder

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Background: An analysis of the relationship between clinical features and allele sharing could clarify the issue of genetic linkage between bipolar affective disorder (BPAD) and chromosome 18q, contributing to the definition of genetically valid clinical subtypes.

Methods: Relatives ascertained through a proband who had bipolar I disorder (BPI) were interviewed by a psychiatrist, assigned an all-sources diagnosis, and genotyped with 32 markers on 18q21-23. Exploratory findings from the first 28 families (n=247) were tested prospectively in an additional 30 families (n=259), and the effect of confirmed findings on the linkage evidence was assessed.

Results: In exploratory analyses, paternal allele sharing on 18q21 was significantly ($P=.03$) associated with a diagnostic subtype, and was greatest in pairs where both siblings had bipolar II disorder (BPII). Prospective analysis confirmed the finding that BPII-BPII sibling pairs showed significantly ($P=.016$) greater paternal allele sharing. Paternal allele sharing across 18q21-23 was also significantly greater in families with at least one BPII-BPII sibling pair. In these families, multipoint affected sibling-pair linkage analysis produced a peak paternal lod score of 4.67 (1-lod confidence interval, 12 centimorgans [cM]) vs 1.53 (1-lod confidence interval, 44 cM) in all families.

Conclusions: Affected sibling pairs with BPII discriminated between families who showed evidence of linkage to 18q, and families who did not. Families with a BPII sibling pair produced an increased lod score and improved linkage resolution. These findings, limited by the small number of BPII-BPII sibling pairs, strengthen the evidence of genetic linkage between BPAD and chromosome 18q, and provide preliminary support for BPII as a genetically valid subtype of BPAD.

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ONE OF the great challenges of research in psychiatry lies in the heterogeneity of the clinical entities.[1] When clinical heterogeneity reflects a diversity of causes at the genetic level, the power to detect susceptibility loci by genetic linkage analysis is greatly reduced. As yet, there is no valid and reliable means of dividing clinical entities such as bipolar affective disorder (BPAD) into genetically simpler subtypes.

In this article, we develop and apply to this problem an approach that defines groups of sibling pairs based on linkage to a putative susceptibility locus, then compares clinical features in the linked and unlinked groups. We hypothesized that differences in clinical features reflect genetic differences, and that linked pairs differ clinically from unlinked pairs. Such an analysis might help to verify and extend the initial linkage finding by defining a clinical subtype that is more clearly linked to the region of interest, and by improving the resolution of the linkage signal.

We applied this approach to the putative linkage between BPAD and markers on chromosome 18q. Linkage to this chromosome was first suggested by Berrettini et al[2] in 1994, with results at the "suggestive" level of genome-wide significance.[3] In an independent sample of 28 pedigrees, Stine et al[4] found evidence of linkage to some of the same markers, but also detected linkage to chromosome 18q21-22, approximately 50 centimorgans (cM) distant. The putative 18q locus was subject to a parent-of-origin effect. The greatest allele sharing was observed for paternally transmitted marker alleles in families with an apparently paternal pattern of illness transmission.

Subsequently, several linkage studies of chromosome 18 markers have been published, each based on different samples, genetic marker maps, and statistical meth-

SUBJECTS AND METHODS

FAMILY ASCERTAINMENT AND EVALUATION

Ascertainment and evaluation methods are detailed elsewhere.[7,20] All families included in this study were ascertained with the following criteria: a proband with a history of bipolar I disorder (BPI); at least 1 additional sibling, or 1 sibling and only 1 parent, with a major affective disorder; and no evidence of major affective disorder in both parental lineages by family history. (Two families in which major affective disorder was discovered in both parental lineages after direct interview, and 3 families whose probands were not felt at final diagnosis to have typical BPI, were included.) Informed consent was obtained from all participants.

Subjects were interviewed by a psychiatrist using the Schedule for Affective Disorders and Schizophrenia-Lifetime Version (SADS-L).[21] Two additional psychiatrists reviewed the interview, family informant data, and any medical records before assigning a best-estimate diagnosis under Research Diagnostic Criteria.[22] The diagnosis of bipolar II disorder (BPII) required a subject's having recurrent major depression as well as hypomanias. Using these methods, we have achieved excellent diagnostic reliability (κ values for BPI, BPII, and recurrent major depression all equalled or exceeded 0.99).

SAMPLES STUDIED

We used a 2-sample design to allow some exploratory data analysis while minimizing chance findings. In the first sample, family set A, we carried out exploratory analyses aimed at formulating a hypothesis as to which clinical features predict allele sharing. Clinical data came from the mania, hypomania, and depression sections of the SADS-L and from the diagnostic subtype (BPI, BPII, recurrent major depression, schizoaffective-manic) assigned by the best-estimate psychiatrists. We subsequently tested the findings from set A in the second independent set B.

Data for set A were originally reported elsewhere.[4] Briefly, it consisted of 286 diagnosed subjects in 28 families. Of these, 59 subjects (21%) had BPI, 49 subjects (17%) had BPII (plus recurrent major depression), and 28 subjects (10%) had recurrent major depression (RUP). A best-estimate diagnosis of "phenotype uncertain" was assigned to 69 subjects (24%), and 81 subjects (28%) were considered unaffected. Based on informativeness for linkage analysis, 247 subjects were selected for genotyping.

Set B was also originally described elsewhere.[7] It consisted of 30 families, and was completed after set A, but before August 1, 1996 (when the data set was "frozen" for analysis upon meeting prior thresholds for statistical power).[7] Of the 300 subjects to whom a best-estimate diagnosis could be assigned, 59 (20%) had BPI, 40 (13%) had BPII plus recurrent major depression, 42 (14%) had RUP, and 6 (2%) had schizoaffective manic disorder. Of the remaining subjects, 70 (23%) were considered unaffected, and 83 (28%) were considered "phenotype uncertain." The 259 most informative subjects were selected for genotyping.

GENOTYPING

Genotyping was performed as described previously.[7] DNA was genotyped by polymerase chain reaction using multiplexed, fluorescent-labeled primers and electrophoresis on an automated sequencer (Perkin Elmer Applied Biosystems Inc, Folster City, Calif) with semiautomated allele scoring. For the exploratory analyses, we used data from the markers D18S41, D18S64, and D18S38 (the 18q markers most strongly linked to BPAD when we originally analyzed these data[4]) typed in 28 families. For the prospective analyses, we used a dense set of 32 markers spanning the region between D18S487 and D18S1095 at a mean

ods. These studies can be interpreted as supporting a pericentromeric locus,[5,6] an 18q21-22 locus,[7,8] both loci,[9,10] other chromosome 18 loci,[11-13] or no compelling chromosome 18 linkage at all.[14-17] Thus, while linkage of BPAD to chromosome 18 seems likely, the evidence in individual samples is modest, and the linkage signals are not well localized, particularly across samples. This is the expected situation with genes of small effect, when significant genetic heterogeneity is present, or when at least some of the results represent false positives.[18,19]

We investigated whether a systematic analysis of the relationship between clinical features and allele sharing could clarify the question of genetic linkage between BPAD and markers on chromosome 18q21-22. We studied the Johns Hopkins/Dana Foundation Bipolar Disorder Pedigrees, a series of multiplex families showing evidence of linkage to chromosome 18q21-22 in prior studies.[4,7] The overall goals were to define the clinical features characteristic of families linked to 18q21-22, verify the prior linkage findings, and improve their resolution as the basis for future work aimed at cloning a susceptibility allele.

RESULTS

EXPLORATORY ANALYSES

Thirty-one variables were analyzed (**Table 1**). Nominally significant differences ($P<.05$) were observed between sharing and nonsharing pairs in occurrence of mania immediately before or after major depression, and in occurrence of mood-congruent psychotic features during major depression. Neither result remained significant after Bonferroni correction. In contrast, a highly significant difference (overall $\chi^2_5=20.89$, $P<.001$) between sharing and nonsharing pairs was observed for diagnostic subtype, which remained significant ($P=.03$) after Bonferroni correction. No significant differences were detected for the other 28 variables.

The significant effect of diagnostic subtype was examined further by direct comparison of allele sharing in sibling pairs grouped into all 6 possible combinations of diagnostic subtype: BPI-BPI, BPI-BPII, BPI-RUP, BPII-BPII, BPII-RUP, and RUP-RUP. Sibling pairs in which both siblings were diagnosed with BPII disorder were most

sex-averaged interval of 2.4 cM. The first analysis of these data has been reported previously.[7]

STATISTICAL METHODS

Exploratory Analyses

Set A genotypes were used to score each sibling pair for unambiguous sharing or nonsharing of marker alleles. The clinical features of sharing vs nonsharing pairs were then compared. For categorical variables with more than 2 categories, all discordant pairs were pooled. Paternal and maternal alleles were analyzed separately, since earlier analyses indicated that on 18q only paternal marker alleles were shared in excess by affected sibling pairs.[4,7] Continuous variables were analyzed by the *t* test; categorical variables, by a maximum-likelihood χ^2 test. The α level of significance was set at .05; multiple comparisons underwent Bonferroni correction. Statistics were calculated using STATISTICA (Release 4.5, StatSoft Inc, Tulsa, Okla).

It was not possible to score allele sharing by all sibling pairs using the single-marker genotype data, so we reanalyzed allele sharing by diagnostic subtype using multipoint haplotypes that take genotypes at adjacent markers into account, and reduce the effect of variable marker informativeness. Multipoint haplotype analyses were performed on both sets A and B using data from the set of 32 markers. Haplotypes were assembled with GENEHUNTER,[23] and sibling pairs were scored based on unambiguous sharing or nonsharing of phased alleles at each marker. Again, paternal and maternal chromosomes were analyzed separately. Haplotypes were also used to edit out probable genotype errors prior to linkage analysis, as detailed elsewhere.[7]

Linkage Analysis

Linkage analysis was performed using the sib_ibd and sib_phase programs in ASPEX.[24] Based on the results of the prior analyses, we partitioned the 58-pedigree sample into those families that had at least 1 BPII-BPII sibling pair (n=16 nuclear families; 1 pedigree was split into 2 nuclear families) and those that did not (n=43 nuclear families). Sex-specific maps were generated using the sib_map program in ASPEX[24]; marker order was determined genetically as described previously,[7] but it cannot be considered definitive for such densely placed markers. Bipolar I disorder and BPII were considered affected phenotypes, and paternal and maternal allele sharing was estimated for all possible affected sibling pairs under an additive genetic model.

The effect of BPII-BPII allele sharing on linkage resolution was assessed by comparing linkage results in the total set of 59 nuclear families with those of the 16 nuclear families with at least 1 BPII-BPII sibling pair. For purposes of comparison, resolution was based on 1-lod confidence intervals. Linkage was tested under the same affection status model (BPI and BPII) in both analyses.

The significance of lod score changes was assessed by generating 5000 samples of 16 nuclear pedigrees randomly selected from the total set, and subjecting each sample to multipoint linkage analysis as described above. A more conservative assessment was based on those random samples with at least 48 sibling pairs, since our actual selection strategy implicitly required at least 48 sibling pairs (16 nuclear families × [2 BPII siblings + 1 BPI proband per family]), and larger samples have more power to detect linkage.

Data Management

Data management was achieved using a relational database system based on PARADOX (versions 5 and 8, Corel Corporation, Ottawa, Ontario), as described elsewhere.[25] The data in this system have undergone rigorous cleaning and editing procedures, with a residual error rate estimated at less than 6 per 10 000 data items.

likely to share an excess of paternal alleles. Fifteen of 15 BPII-BPII pairs (100%) shared paternal alleles identical by descent (IBD), compared with 22 of 30 pairs (73%) for BPI-BPII sibling pairs and IBD proportions close to the expected 50% for each of the 4 other types of affected sibling pairs. This result was confirmed by multipoint haplotype analysis. Again, paternal allele sharing was significantly associated with a diagnostic subtype (overall $\chi^2_5=16.16$, $P=.007$) and was greatest in BPII-BPII sibling pairs (**Figure 1**A).

PROSPECTIVE ANALYSIS

Based on these results, we formulated the hypothesis that BPII-BPII sibling pairs share paternal alleles on 18q21 more often than the other types of sibling pairs. Subsequently, this hypothesis was tested in an independent set of 30 families using multipoint data (Figure 1B). This indicated that BPII-BPII sibling pairs shared 9 of 11 paternal marker alleles (82% IBD), which was significantly more than the 71 of 129 (55% IBD) proportion of allele sharing observed in the other types of affected sibling pairs taken together (Fisher exact test=0.016). An apparent decrease in paternal allele sharing by RUP-RUP pairs was based on only 8 pairings and was not significant.

EFFECT ON LINKAGE EVIDENCE

Inspection of allele sharing in each family revealed that BPI siblings shared paternal alleles with BPII-BPII sibling pairs in the same family. We therefore hypothesized that entire families with 1 or more BPII-BPII sibling pairs would show genetic linkage to 18q, while other families would not. The results are presented in **Table 2**.

The BPII sibling pair families demonstrated linkage to several 18q markers. In these families, there was highly significant ($P\leq.005$) linkage to paternal alleles at five 18q21 markers, near the markers tested in the initial clinical analyses, and to 4 more distal markers. The evidence for linkage peaked at D18S346 in 18q21 (86.0% IBD; $\chi^2_1=22.35$, $P=.000002$) and again distally at D18S1106 in 18q22-23 (80.8% IBD; $\chi^2_1=19.69$, $P=.000009$).

Table 1. Clinical Variables Analyzed*

Feature/Variable	No. of Pairs†	Feature/Variable	No. of Pairs†
Age at first outpatient treatment	25	Diagnostic subtype	94
No. of episodes of mania	13	Mood congruent psychotic features with mania	25
Age at first episode of mania	13	Felt guilty during major depression	82
No. of episodes of major depression	28	Problem concentrating during major depression	81
Age at first major depression	28	Suicidal thoughts/behavior during major depression	82
No. of episodes of hypomania	22	Appetite change during major depression	40
Age at first episode of hypomania	22	Sleep disturbance during major depression	45
Overactive during mania	36	Low energy during major depression	81
Overtalkative during mania	33	Low interest during major depression	82
Racing thoughts during mania	33	Psychomotor changes during major depression	76
Grandiose during mania	34	Mania immediately preceded/followed depression	82
Needed less sleep during mania	34	Depression associated with pregnancy	38
Distractable during mania	34	Depression associated with menopause	29
Poor judgment during mania	34	Depression followed somatic treatment	78
Felt depressed immediately before or after mania	25	Mood congruent psychotic features with depression	81
Mania followed somatic treatment	25		

*Age at first inpatient treatment was dropped since only 9 pairs could be scored for this feature.
†No. of pairs indicates the number of affected sibling pairs contributing data for each feature or variable.

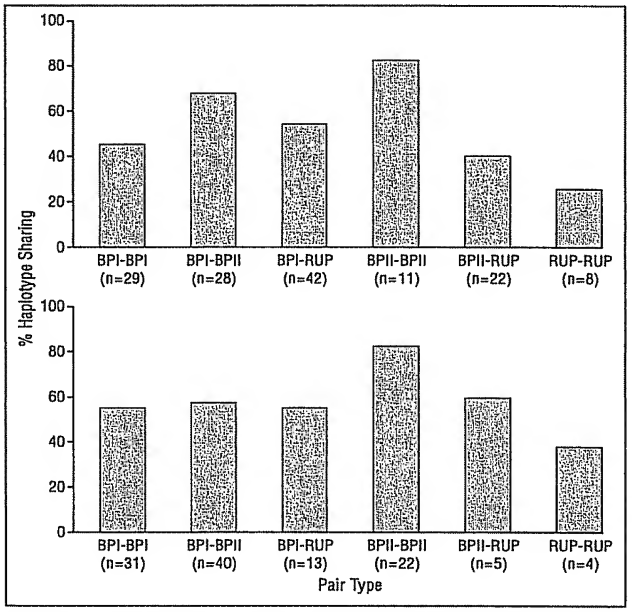

Figure 1. Paternal haplotype sharing across D18S38 (58 families) in affected sibling pairs, grouped by pair type. Data for families from set A are given in the top half of the figure, and those from set B are provided in the bottom half. The total number of scored pairs ([n*{n-1}]/2) is indicated under each pair type on the x-axis. BPI indicates bipolar I disorder; BPII, bipolar II disorder; and RUP, recurrent unipolar depression.

Families of BPII sibling pairs accounted for essentially all of the evidence of linkage to 18q previously observed in this pedigree sample. No evidence of linkage was detected in the 43 families with no BPII-BPII sibling pairs, even though these families had a larger total number of affected sibling pairs and would display more evidence of linkage if it existed. When paternal allele sharing in the 2 sets of families was directly compared (Table 2), there was significantly ($P \leq .01$) increased sharing at 12 markers in BPII sibling pair families; the most significant difference (80.8% vs 47.2% IBD; $\chi^2_1 = 20.19$, $P < .001$) was observed at D18S1106. Little evidence of linkage to maternal alleles was seen in either group of families. These data are consistent with one or more 18q loci linked to BPAD in BPII sibling pair families.

EFFECT ON LINKAGE RESOLUTION

The linkage resolution was substantially improved in families with a BPII-BPII pair (**Figure 2**). In these families, the 1-lod confidence interval for linkage spanned approximately 12 cM (sex averaged), compared with a confidence interval of more than 44 cM (sex averaged) for all 58 families. This approximately 3-fold improvement in linkage resolution is consistent with published simulations,[19] and it implies a substantial decrease in genetic heterogeneity. Also consistent with a decrease in heterogeneity was an increase in the peak paternal lod score, from 1.53 in the total sample to 4.67 in the 16 BPII sibling pair families. This occured despite a reduction in sample size from 164 to 81 affected sibling pairs.

The observed increase in lod score was highly significant by asymptotic theory ($\chi^2_1 = 18.79$, $P < .001$) and by simulation. A paternal lod score greater than 4.67 was observed 22 times in 5000 simulations — equivalent to an empirical P value of .004. The empirical P value remained significant ($P = .01$) when the estimate was restricted to random samples containing at least 48 sibling pairs (see the "Subjects and Methods" section).

COMMENT

In both exploratory and prospective analyses performed on independent sets of families, affected sibling pairs with BPII were distinguished between families who showed evidence of genetic linkage to chromosome 18q and families who did not. The families with BPII-BPII pairs demonstrated significantly higher allele sharing at several 18q markers, accounting for essentially all the evidence of linkage to this region previously observed in this sample. Furthermore, families with BPII-BPII pairs substantially improved the peak lod score and the resolu-

Table 2. Point-Wise Identical by Descent Sharing at 18q Marker Loci*

	16 Families With at Least 1 BPII-SPII Sibling Pair						43 Families Lacking a BPII-BPII Sibling Pair							
	Paternal			Maternal			Paternal			Maternal			BPI vs BPII	
Locus	% IBD	χ^2	*P*	% IBD	χ^2	*P*	% IBD	χ^2	*P*	% IBD	χ^2	*P*	χ^2	*P*
D18s487	59.10	0.73	.39	46.40	0.14	.71	54.50	0.18	.67	55.00	0.20	.65	0.09	.76
D18s41	62.30	3.19	.07	54.80	0.29	.59	48.80	0.02	.89	52.10	0.08	.78	1.74	.19
D18s849	58.30	1.67	.20	47.60	0.14	.71	49.20	0.02	.89	49.20	0.02	.89	1.04	.31
D18s1103	62.50	2.50	.11	43.60	0.64	.42	46.20	0.38	.54	58.10	1.61	.20	2.65	.10
D18s1155	55.60	0.44	.51	73.70	4.26	.04	50.00	0.00	1.00	50.00	0.00	1.00	0.24	.62
D18s64	78.30	19.27	<.001	48.40	0.06	.81	50.00	0.00	1.00	37.50	3.00	.08	10.02	<.001
D18s38	75.00	15.00	<.001	47.20	0.17	.68	63.20	2.63	.10	46.30	0.30	.58	1.57	.21
D18s1134	63.40	2.95	.09	60.00	1.00	.32	57.50	0.90	.34	56.20	0.50	.48	0.30	.59
D18s60	92.00	17.64	<.001	40.00	1.00	.32	50.00	0.00	1.00	46.20	0.23	.63	11.46	<.001
D18s1147	73.20	12.07	<.001	50.80	0.02	.89	49.30	0.01	.92	47.50	0.15	.70	7.31	<.001
D18s346	86.00	22.35	<.001	47.40	0.11	.74	55.60	0.56	.45	45.80	0.33	.57	9.82	<.001
D18s68	65.90	4.45	.03	52.20	0.09	.77	49.20	0.02	.89	53.10	0.25	.62	2.91	.09
D18s814	64.90	5.07	.02	46.80	0.26	.61	50.00	0.00	1.00	53.60	0.29	.59	2.33	.13
D18s55	66.00	4.79	.03	48.80	0.02	.89	53.70	0.37	.54	56.60	0.92	.34	1.70	.19
D18s465	61.30	1.58	.21	45.50	0.18	.67	52.50	0.15	.70	49.20	0.02	.89	0.63	.43
D18s382	64.30	1.14	.29	90.50	13.76	<.001	52.00	0.04	.84	53.30	0.13	.72	0.55	.46
D18s1131	69.80	6.72	.01	51.50	0.03	.86	54.90	0.49	.48	50.80	0.02	.89	2.18	.14
D18s61	70.40	8.96	.003	49.10	0.02	.89	41.90	1.61	.20	51.60	0.06	.81	9.44	.002
D18s1125	67.40	5.57	.018	48.10	0.08	.78	42.20	1.09	.30	47.10	0.12	.73	5.82	.016
D18s485	73.30	6.53	.011	54.20	0.33	.57	40.00	1.80	.18	55.40	0.64	.42	8.04	.005
D18s1106	80.80	19.69	<.001	48.30	0.07	.79	37.30	3.31	.07	47.20	0.17	.68	20.19	<.001
D18s541	64.70	2.94	.09	57.90	0.47	.49	47.90	0.08	.78	52.50	0.15	.70	2.27	.13
D18s870	58.30	0.67	.41	53.30	0.20	.65	50.00	0.00	1.00	45.50	0.36	.55	0.38	.54
D18s1161	66.70	3.67	.06	50.00	0.00	1.00	36.70	3.45	.06	41.00	1.26	.26	7.07	.008
D18s1112	73.90	5.26	.022	52.90	0.06	.81	48.90	0.02	.89	42.90	0.43	.51	3.90	.048
D18s1121	100.00	7.00	.008	77.80	2.78	.10	53.80	0.08	.78	36.40	0.82	.37	4.62	.032
D18s844	68.00	3.24	.07	50.00	0.00	1.00	40.90	0.73	.39	45.50	0.18	.67	3.47	.06
D18s1115	62.20	2.69	.10	51.00	0.02	.89	42.10	0.95	.33	41.20	0.53	.47	3.35	.07
D18s554	72.70	9.09	.003	44.00	0.36	.55	42.00	1.28	.26	43.90	0.61	.43	8.99	.003
D18s462	70.20	7.68	.006	50.00	0.00	1.00	37.50	2.50	.11	51.90	0.07	.79	9.35	.002
D18s1122	74.30	8.26	.004	56.40	0.64	.42	43.90	0.61	.43	49.10	0.02	.89	7.15	.008
D18s1095	66.10	6.12	.013	64.30	1.14	.29	40.00	1.60	.21	51.90	0.07	.79	6.58	.01

*BPII indicates bipolar II disorder; BPI, bipolar I disorder; and IBD, identical by descent. All paternal and maternal percentage IBD values are proportions of sibling pairs sharing alleles identical by descent. All *P* values are estimated with 1 *df*.

tion of the multipoint linkage analysis. These results strengthen the evidence that a gene important in BPAD resides on chromosome 18q and provides preliminary support for BPII as a genetically valid subtype of BPAD.

This study has several strengths. All subjects were interviewed and diagnosed by psychiatrists. Perhaps as a result of this, we achieved high interrater reliability. In particular, the diagnosis of BPII disorder showed a κ score of 0.99 at the best-estimate level in this sample. The clinical data were managed using stringent error prevention, detection, and correction procedures,[25] and the genotype data were derived from a dense microsatellite marker map. The potential effect of multiple comparisons on type I error was minimized by a 2-sample design and by assessing the significance of the lod score change through simulation.

The major weakness of this study is the small number of BPII-BPII sibling pairs in each set of families. Although to our knowledge this is one of the largest BPAD family samples studied to date, with 586 subjects in 58 pedigrees, set A contained only 15 independent BPII-BPII pairs, and set B contained only 11. Our linkage results are based on all 81 affected sibling pairs in the 16 BPII sibling pair families, not just the BPII-BPII pairs; however, even this sample is not large by current standards. Stratification can be a valuable strategy when it results—as in this study—in increased allele sharing[26]; however, the shrinking of comparison groups is an inevitable consequence of subdividing the affected phenotype in an attempt to define more genetically homogeneous groupings. One could avoid stratification, and perform instead a covariate-based linkage analysis, a method that is now becoming feasible.[27,28] Such a method should also ideally allow the simultaneous assessment of many clinical variables with allele-sharing, thus minimizing multiple comparisons. Another weakness of this study is the reliance on the retrospective self-report of clinical symptoms by subjects. The effect of any misreporting was minimized by seeking corroborative data in medical records and in the reports of family informants.

The more severe forms of a phenotype are traditionally considered easier to map by linkage analysis. Accordingly, some authorities have recommended using only BPI cases in linkage studies.[14,29,30] Our data suggest that this strategy may not be optimal for detecting genetic linkage to 18q, even in families ascertained through BPI pro-

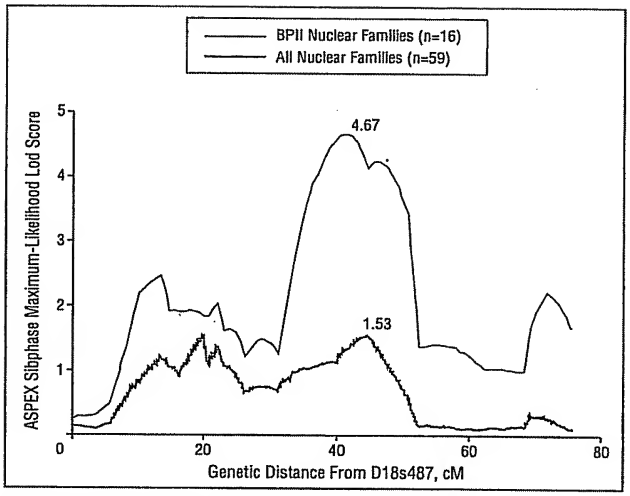

Figure 2. Genetic linkage results for 32 markers on chromosome 18q21-23. The analysis was performed in the total set of 59 nuclear families, and in the 16 nuclear families with at least 1 BPII-BPII (bipolar II disorder) sibling pair. For both analyses, BPI (bipolar I disorder) and BPII subjects were considered affected. The sex-averaged genetic distance is shown on the x-axis, and the multipoint maximum-likelihood paternal lod score is shown on the y-axis. CM indicates centimorgan.

bands. In our sample, the siblings with BPII contributed substantially to the detection of linkage, and they clustered in families that had the greatest evidence of linkage to 18q. Bipolar I disorder may actually represent complications of a milder, genetically less complex disorder, as we have suggested previously.[31]

Parent-of-origin effects encompass an important and growing class of clinical genetic phenomena, wherein the phenotype in the offspring depends on the sex of the transmitting parent.[32] We have previously reported that the parent of origin influences the linkage of BPAD to chromosome 18q. In the same 28 families that constitute set A, Stine et al[4] found greater lod scores in families with apparently transmitting fathers — an effect that was most striking for 18q21 markers. In the same 30 families that constitute set B, McMahon et al[7] observed the largest IBD scores for paternally transmitted marker alleles, but the excess allele sharing was not confined to families with apparently transmitting fathers. Thus, the paternal parent-of-origin effect seems to be robust when based on observed transmission of marker alleles, but not when based on apparent transmission of illness. This may reflect uncertainty in the classification of families by apparent pattern of illness transmission, hidden bilineality, or other factors.

In order to take the prior findings on 18q into account while avoiding these uncertainties, we chose to base the present analysis on observed transmission of marker alleles. We again found robust evidence of linkage to paternal, but not maternal alleles. Only 4 of the 16 families with BPII sibling pairs had an apparently transmitting father. This is consistent with our conclusion that the clustering of BPII-BPII sibling pairs is the key feature of families linked to18q in this sample. The importance of paternally transmitted marker alleles remains, and perhaps implicates genomic imprinting or other mechanisms.

MacKinnon et al[33] evaluated the effect of panic attack comorbidity on linkage of BPAD to 18q. Post hoc analyses of set A indicated that the linkage evidence was strongest in families where the proband had panic attacks. This could not be adequately tested in set B, since only 2 families had a proband with panic attacks. We cannot rule out that another factor correlated with BPII, and perhaps also with panic attacks, may account for our findings, and we have not tested the effect of BPII on linkage elsewhere in the genome. Nevertheless, diagnostic subtype was the only 1 of 31 variables we analyzed that successfully discriminated linked and unlinked families in our sample.

The peak paternal lod score of 4.67 that we observed in the BPII sibling pair families should be viewed with caution. This lod score was obtained after past analyses of the same data sets that evaluated linkage in a variety of ways.[4,7,34-35] Our simulation studies indicate that the increase in lod score that was observed in the BPII sibling pair families would rarely be seen by chance alone. Furthermore, the increase in lod score was not diffuse, but it was focused at a few adjacent markers, thus increasing the resolution of the linkage signal and fulfilling one of the chief aims of this study.

Friddle et al[34] published a genome-wide linkage study of BPAD, using 50 of the 58 families included herein. That study found little evidence of linkage to 18q or any other locus, either by single-locus nonparametric analyses or by a 2-locus heterogeneity analysis. Several important differences with the present study may account for the discrepant results. Friddle et al employed a less dense marker map on 18q21-23 (an approximately 9-cM mean interval, compared with 2.4 cM in the present study), and the marker data they analyzed were fully informative in only about half as many sibling pairs.[34] Marker density and sample size can be critical factors in detecting alleles of modest effect.[35] In addition, Friddle et al[35] did not analyze their results by parent of origin. Since most of the positive linkages between BPAD and 18q report a parent of origin effect, this may be an important factor in detecting linkage to this region. Friddle et al also failed to detect linkage under their heterogeneity model, which assumed 2 major loci that each accounted for linkage in at least half of the families.[34] This failure would not be surprising if, as we now conclude, the 18q linkage is primarily confined to BPII sibling pair families, which constitute much less than half (27%) of this sample.

Our results offer a potential explanation for the apparently inconsistent results of previous chromosome 18q linkage studies and point to a strategy for replication. Future studies should take careful account of the families with BPII-BPII sibling pairs and should analyze linkage separately for paternal and maternal marker alleles. By decreasing heterogeneity, this approach may lead to more consistent results that could ultimately clarify the complex molecular anatomy of BPAD, moving us closer to the identification of susceptibility alleles.

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Exhibit C

Am. J. Hum. Genet. 61:1397–1404, 1997

Linkage of Bipolar Affective Disorder to Chromosome 18 Markers in a New Pedigree Series

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Summary

Several groups have reported evidence suggesting linkage of bipolar affective disorder (BPAD) to chromosome 18. We have reported data from 28 pedigrees that showed linkage to marker loci on 18p and to loci 40 cM distant on 18q. Most of the linkage evidence derived from families with affected phenotypes in only the paternal lineage and from marker alleles transmitted on the paternal chromosome. We now report results from a series of 30 new pedigrees (259 individuals) genotyped for 13 polymorphic markers spanning chromosome 18. Subjects were interviewed by a psychiatrist and were diagnosed by highly reliable methods. Genotypes were generated with automated technology and were scored blind to phenotype. Affected sib pairs showed excess allele sharing at the 18q markers D18S541 and D18S38. A parent-of-origin effect was observed, but it was not consistently paternal. No robust evidence of linkage was detected for markers elsewhere on chromosome 18. Multipoint nonparametric linkage analysis in the new sample combined with the original sample of families supports linkage on chromosome 18q, but the susceptibility gene is not well localized.

Introduction

Reports of linkage in psychiatric disorders have proliferated in recent years, with few replicated findings. This has promoted skepticism about all linkage findings in psychiatric disorders, despite the acknowledgment that the replication of true linkage findings for psychiatric disorders will be a difficult task (Risch and Botstein 1996). The range of phenotypes that share genetic risk factors, the number of genes involved, and the mode of inheritance are all unknown. Nevertheless, until susceptibility genes are identified, replication remains the most important indicator of valid linkage findings.

Linkage studies of bipolar affective disorder (BPAD) have now led to positive findings at nearby loci in two or more samples for chromosome 21 and chromosome 18. Detera-Wadleigh et al. (1996) published support for a prior linkage finding on chromosome 21q (Straub et al. 1994). For chromosome 18, Berrettini et al. (1994) reported significant evidence of linkage to markers in the pericentromeric region, with a peak identical by descent (IBD) score of .58 ($P = .0004$), although parametric LOD scores in the total sample were all negative. Our group (Stine et al. 1995) followed up these results in a set of 28 families selected for the presence of affected phenotypes in only one parental lineage (Simpson et al. 1992). Our findings showed a peak in the linkage statistic on 18p11 (IBD = .64, $P = .0003$), which overlapped with the previously reported findings, and another peak 40 cM distant on 18q21 (IBD = .57, $P = .02$) (Stine et al. 1995). These results appeared to strengthen those of Berrettini et al. (1994), but we could not draw firm conclusions as to either the number of loci involved or their precise location. Three other groups have since published supportive evidence for an 18q locus in BPAD (Coon et al. 1996; de Bruyn et al. 1996; Freimer et al. 1996). These studies all report LOD scores in the range of 1–2, although de Bruyn et al. found one 18q22 marker linked at the $P < .0007$ level in an affected-sib-pair analysis and Freimer et al. obtained a maximum LOD score of 4.06 for an 18q23 marker in a "joint linkage and association analysis."

The reports by Berrettini et al. (1994) and by Stine et al. (1995) both suggest loci that confer a relative risk <3 (Risch 1987). Linkage replication for loci conferring such a modest increased disease risk may be a considerable challenge. Indeed, several published reports have failed to detect linkage between BPAD and chromosome 18 markers (Kelsoe et al. 1995; Maier et al. 1995; Pauls et al. 1995; Smyth et al. 1995; Detera-Wadleigh et al.

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1997). A locus conferring a small relative risk may require larger samples than those used in these reports, to ensure adequate power for replication (Suarez 1994). Even when replication is achieved, the results may not correspond well to the site of the original findings, since the peak of the linkage statistic for complex phenotypes may not correspond to the site of the actual disease locus (Kruglyak and Lander 1995).

The linkage findings on chromosome 18 are further complicated by an apparent parent-of-origin effect. In our previous sample, most of the evidence for linkage derived from families with affected phenotypes in only the paternal lineage and from marker alleles transmitted on the paternal chromosome. Heterogeneity reflected in the sex of the apparently transmitting parent was suggested by our earlier clinical studies (McMahon et al. 1995), in which an excess of affected maternal relatives and a higher-than-expected proportion of families with no paternal transmission were observed. However the post hoc application of the parent-of-origin effect to the linkage analysis introduced additional df and underscored the need for replication. Other groups have since shown a paternal effect on the linkage evidence in the pericentromeric region of chromosome 18 (Gershon et al. 1996; Nöthen et al. 1996), but, to date, no confirmatory reports of a paternal effect on 18q have appeared.

In order to test the hypothesis that BPAD is linked to markers on chromosome 18, we studied 13 highly polymorphic markers spanning the chromosome in a new, 30-family set ascertained and analyzed in a manner similar to that used for the first sample of families. The results support our previously reported linkage of BPAD to chromosome 18q, but the parent-of-origin effect is less consistent than that in the original sample, and no robust evidence of linkage was detected for markers elsewhere on chromosome 18 in this relatively small set of families.

Subjects and Methods

Family Ascertainment and Evaluation

Clinics and inpatient units in Baltimore and Iowa City were screened for treated probands with bipolar I disorder (BPI) as described elsewhere (Simpson et al. 1992). Probands who, on the basis of family history, had either two or more two sibs with a major affective disorder or one sib and only one parent with a major affective disorder were enrolled for study. Informed consent was obtained from all participants.

Probands and their relatives were interviewed by a psychiatrist trained in the use of the Schedule for Affective Disorders and Schizophrenia—Lifetime Version, a semistructured psychiatric interview of established reliability and validity (Endicott and Spitzer 1978). Every effort was made to interview the parents, aunts, uncles, and grandparents, so that any affected phenotypes could be detected in both parental lineages. Such "bilineal" families were not evaluated further. Later, the interview data were reviewed, along with family-informant data and any available medical records, by two additional psychiatrists who assigned a best-estimate diagnosis based on research diagnostic criteria (Spitzer et al. 1975). When the best-estimate reviewers could not agree that a subject was affected with BPAD, or when a subject was assigned phenotypes that have been related to BPAD in family studies, that subject was classified as "phenotype uncertain" (for details, see Simpson et al. 1992). Our best-estimate diagnoses have very high reliability (see Stine et al. 1995).

The 23 completely evaluated families meeting ascertainment criteria as of August 1, 1996, were selected for genotyping. These families consisted of 9 "paternal" pedigrees (in which the proband's father or one of his sibs or parents is affected) and 14 "maternal" pedigrees (in which the proband's mother or one of her sibs or parents is affected), as defined elsewhere (see Stine et al. 1995). This set of families alone did not fulfill the threshold of 90% power to detect linkage, which we had set as a requirement for commencement of the confirmation study. Therefore we selected for genotyping seven additional families, before commencing genotyping. Since our previous linkage findings on 18q had suggested a paternal parent-of-origin effect, the additional families were selected for a paternal pattern of transmission. Although clinically similar to the other families, these families did not meet the original ascertainment criteria, for one or more of the following reasons: (1) they were considered "bilineal," since (in addition to the affected paternal relative) the proband's mother or a maternal aunt, uncle, or grandparent was found to have recurrent unipolar disorder (RUP) (two families); (2) the mother, although reportedly unaffected, could not be interviewed (one family); (3) the proband was not felt by the best-estimate diagnosticians to have typical BPI (three families); or (4) only an affected sib pair was available for study (one family).

In this total sample, of the 251 subjects for which a best-estimate diagnosis could be assigned, 56 (22.3%) were diagnosed with BPI, 38 (15.1%) with bipolar II disorder (BPII) plus recurrent major depression, 38 (15.1%) with RUP, and 4 (1.6%) with schizoaffective manic disorder (SAM). Of the remaining subjects, 51 (20.3%) were considered unaffected, and 56 (22.3%) were classified as phenotype uncertain. This is similar to the distribution of diagnoses in the original family sample (Stine et al. 1995). No best-estimate diagnosis could be assigned for eight subjects, because of inadequate phenotype data. These subjects and those assigned a best-estimate diagnosis of phenotype uncertain were

considered phenotype unknown for the purposes of linkage analyses.

Marker Selection

Two sets of markers were genotyped: the first set of 13 markers, spanning the chromosome, was selected in order to test confirmation of linkage on chromosome 18. The second set was selected with the goal of improving localization by saturation of the region surrounding the linkage finding on 18q. For the confirmation study, we chose to genotype five of the markers that had given the strongest linkage evidence in previous studies (markers D18S53, D18S37, D18S41, D18S64, and D18S38; Berrettini et al. 1994; Stine et al. 1995). In addition, eight markers covering other regions of the chromosome were selected from the Weber 6 set (Research Genetics), in order to take advantage of automated genotyping methods. For the subsequent 18q saturation study, 41 polymorphic markers between D18S41 and D18S70 were selected. Primer sequences were obtained from the Genome Database.

DNA Preparation and Genotyping

Genotyping was performed at laboratories at Johns Hopkins University and at Sequana Therapeutics. At Sequana, the methods employed have been detailed by Hall et al. (1996). In brief, lymphoblastoid-cell-line DNA was prepared in standard concentration, and samples were assembled into 96-well master plates. Each plate lot was tested for quality by a panel of 10 microsatellite markers.

PCR reactions were performed in an automated manner by a robotic workstation. Each well contained a 20-μl total-reaction volume. Touchdown PCR was performed in PTC-100 Peltier-effect thermal cyclers (MJ Research) by use of a standard protocol (Hall et al. 1996).

Multiplexed PCR products were electrophoresed on ABI Prism 377 sequencers. The gel-file output was checked for correct tracking, and automated size-standard-validation software checked the allele size determination. A control DNA sample was run on each gel, in order to correlate allele sizes among gels. Genotypes were read by GeneScan 2.1fc2 and Genotyper 1.1 programs. Once allele sizes for each microsatellite marker had been determined for all DNA samples in the study, arbitrary allele numbers were assigned, and the data were checked for correct Mendelian-inheritance patterns. At this stage, data from four markers in the saturation set were dropped, because of inconsistent binning.

Similar DNA-preparation and genotyping methods were employed at Johns Hopkins, except that DNA concentrations were determined spectrophotometrically. PCR reactions were performed manually and were optimized for each pair of primers. Initial allele scoring was done automatically and was checked manually. Genotypes were binned and tested for inheritance by use of GAS 2.0 (Genetic Analysis System, version 2.0; Young 1995). Alleles that fell outside the fixed bin sizes or that did not segregate were rechecked; the data from that portion of the pedigree were deleted if discrepancies could not be resolved.

Statistical Methods

Linkage was tested by a limited number of analytic methods, in order to avoid the problem of multiple tests. Nonparametric methods were chosen, since they are more powerful when genetic parameters are unknown.

Power analyses indicated that our sample had >90% power to detect linkage in affected sib pairs, at the $P \leqslant .01$ level, when subjects with BPI, BPII, RUP, or SAM were included in the analysis. The software package SLINK was used to generate simulated data on the basis of the pedigree structure, phenotype information, and genotype availability of the 30 pedigrees. A dominant model and a marker with six equally frequent alleles linked to the disease locus at a recombination fraction (θ) of .01 in all families was assumed. One thousand replicates of the simulated data were then analyzed by SIBPAL.

Since some cases of RUP ascertained through BPI probands may have distinct etiologies (Blacker and Tsuang 1993; McMahon et al. 1994), we also performed analyses excluding RUP subjects. On the basis of our simulations, the sample excluding RUP subjects had 89.5% power to detect linkage, at the $P \leqslant .01$ level.

The SIBPAL module of SAGE was used to test the null hypotheses of 50% allele sharing by affected sib pairs, in the total sample and in paternal pedigrees. The SIBDES module of GAS was used to test the null hypothesis of 50% allele sharing, by affected sib pairs, of 18q markers on paternally transmitted chromosomes. P values were adjusted for nonindependent sib pairs (Hodge 1984).

To improve our estimate of linkage localization, the data from 32 of the markers genotyped in the total sample of 58 families were analyzed by the all-relative-pair option in GENEHUNTER (Kruglyak and Lander 1995). For this analysis, we selected the most informative markers—that is, those with the fewest missing genotypes. GENEHUNTER haplotypes identified 11 single-marker apparent double recombinants in six families; genotypes from these markers were zeroed out in these families. In our sample, allele frequencies were determined by use of subjects without parents.

For the confirmation study, map order was determined with CRIMAP and agrees with the order on published maps. For the saturation study, the order of all such

Table 1

Results of Affected-Sib-Pair Analyses for Sibs Affected with BPI, BPII, SAM, or RUP, in 30 Families

Marker	Cumulative Sex Average (cM)	BPI/BPII/SAM			BPI/BPII/SAM/RUP		
		No. of Pairs	IBD[a]	P[b]	No. of Pairs	IBD[a]	P[b]
D18S63	0	69	.45	NS	150	.48	NS
D18S452	12.3	69	.51	NS	147	.53	NS
D18S53	31.3	72	.43	NS	154	.48	NS
D18S37	34.3	74	.50	NS	152	.52	NS
D18S847	45.3	69	.50	NS	149	.52	NS
D18S548	60.0	64	.50	NS	126	.56	.0013
D18S487	71.0	73	.52	NS	156	.51	NS
D18S41	73.9	62	.52	NS	130	.52	NS
D18S849	77.4	68	.51	NS	149	.51	NS
D18S64	81.3	74	.53	NS	157	.52	NS
D18S38	84.5	73	.55	.0584	154	.54	.0352
D18S541	99.1	72	.62	.0003	154	.54	.0670
D18S844	111.3	74	.53	NS	157	.53	NS

NOTE.—All P values <.1 are shown.

[a] Mean proportion of marker alleles shared IBD (by SIBPAL).

[b] NS = not significant.

closely spaced markers could not be determined unambiguously in this data set. However, the order of those markers which could be placed at 1,000:1 odds agrees with the standard chromosome 18 reference map (Collins et al. 1996).

Results

Two-Point Affected-Sib-Pair Analyses

Results are presented in table 1. Excess allele sharing by affected sib pairs was observed at the 18q markers D18S38 and D18S541. At D18S541, sib pairs affected with BPI, BPII, or SAM shared a mean 62% of alleles IBD ($P = .0003$). When RUP sibs were included, excess allele sharing was also seen at one 18p marker, D18S548 (IBD = .56, $P = .0013$), but with no evidence of linkage at the flanking markers D18S847 and D18S487, 15 cM and 11 cM distant, respectively.

As in the previous sample (Stine et al. 1995), the greatest excess allele sharing was seen for paternally transmitted chromosomes (table 2). At D18S541, 77.9% of paternal alleles ($P = .015$) and 59.5% of maternal alleles (P not significant) were shared IBD by sib pairs affected with BPI, BPII, or SAM. In contrast to the previous sample, evidence of linkage appeared greater in "maternal" pedigrees, although stratification by parent of origin caused the sample sizes to become very small (table 2). Similar results were seen when RUP sibs were included (data not shown).

Multipoint Affected-Relative-Pair Analysis

To improve localization of the linkage finding on 18q, genotype data from a total sample of 58 pedigrees, consisting of the original, 28-pedigree set described elsewhere (Stine et al. 1995) and the new, 30-pedigree set used for the replication study described above, were analyzed. The results for the narrower definition of the affected phenotype are reported, but they did not differ substantially when RUP sibs were included.

The multipoint results were consistent with the two-point findings (fig. 1). The peak nonparametric LOD (NPL) score was observed at D18S38 (2.84; $P < .0019$). Although strongly supportive of linkage, this result was not well localized. The NPL score is associated with $P < .01$ over an interval of ~14 cM.

Discussion

In order to test our previous linkage findings for BPAD on chromosome 18, we have studied 13 polymorphic markers spanning the chromosome in a new, 30-pedigree series ascertained and analyzed in a manner similar to that used with the first sample of families. The results support our previously reported linkage of BPAD to chromosome 18q, but the parent-of-origin effect is less consistent than that in the original sample, and no robust evidence of linkage was detected for markers elsewhere on chromosome 18 in this relatively small set of families.

Lander and Kruglyak (1995) have proposed criteria for "suggestive," "significant," and "confirmed" linkages in a genomewide scan. These statistical thresholds may be too conservative (Sawcer et al. 1997), and their application to complex phenotypes has been criticized since "they might not provide an accurate picture of the multifactorial situation" (Witte et al. 1996, p. 355). By the Lander and Kruglyak (1995) thresholds, our results

Table 2

Results of Affected-Sib-Pair Analyses for Sibs Affected with BPI, BPII, or SAM

	Total Sample						Families Stratified on the Basis of Parent of Origin					
	Paternal Chromosome			Maternal Chromosome			Paternal Families			Maternal Families		
Marker	No. of Pairs[a]	IBD[b]	P[c]	No. of Pairs[a]	IBD[b]	P[c]	No. of Pairs	IBD[d]	P[c]	No. of Pairs	IBD[d]	P[c]
D18S63	30.1	.45	NS	31.8	.42	NS	26	.39	NS	22	.49	NS
D18S452	33.1	.52	NS	31.3	.47	NS	27	.44	NS	21	.63	.022
D18S53	26.4	.45	NS	27.4	.42	NS	29	.41	NS	22	.40	NS
D18S37	12.8	.41	NS	14.4	.55	NS	30	.51	NS	23	.50	NS
D18S847	22.4	.51	NS	12.5	.53	NS	26	.47	NS	22	.50	NS
D18S548	15	.47	NS	15.5	.52	NS	28	.50	NS	20	.49	NS
D18S487	25.2	.53	NS	27.4	.47	NS	30	.47	NS	22	.50	NS
D18S41	18.8	.47	NS	16.2	.58	NS	23	.46	NS	23	.50	NS
D18S849	32.7	.46	NS	33.9	.54	NS	28	.51	NS	22	.56	NS
D18S64	28.6	.57	NS	26.2	.36	NS	30	.47	NS	23	.61	.028
D18S38	25.6	.68	.054	25.4	.53	NS	30	.51	NS	22	.61	.01
D18S541	18.9	.78	.015	25.8	.59	NS	29	.57	NS	22	.64	.024
D18S844	26.2	.56	NS	28.4	.43	NS	30	.56	NS	23	.50	NS

NOTE.—All P values <.1 are shown.

[a] Informative affected sib pairs, weighted by the method of Hodge (1984).

[b] Proportion of alleles shared IBD in informative matings (by GAS).

[c] NS = not significant.

[d] Mean proportion of marker alleles shared IBD (by SIBPAL).

would not qualify as a "confirmed" linkage, even though our affected-sib-pair results reach the confirmatory ($P \leqslant .01$) threshold of significance at D18S541. This is because the 18q linkage results in the first, 28-family sample did not reach the Lander and Kruglyak (1995) threshold for significant linkage ($P \leqslant 2.2 \times 10^{-5}$), except when paternally transmitted chromosomes in paternal families were analyzed, introducing additional df. Nevertheless, our results in this independent sample of 30 families again detect evidence of linkage to 18q and, in this sense, support our original finding.

Has the overall evidence of linkage on 18q been strengthened by the results in the new sample—and, if so, by how much? Significant evidence of linkage between BPAD and 18q markers is seen in both the original, 28-family sample and the new, 30-family sample reported herein, but the peak IBD score in the original sample (81% of paternal alleles shared; $P = .00002$) occurred at D18S41, whereas the peak IBD score for the new sample (78% of paternal alleles shared; $P = .015$) occurred at D18S541, ~25 cM distant from D18S41. This variation in the linkage peak is not resolved in the multipoint analysis, so it does not appear to result solely from variation in marker informativeness (fig. 1). Direct comparisons between individual markers in different samples may be problematic, since, in complex phenotypes, different sets of families contain different proportions of cases that are phenocopies or genetically heterogeneous with respect to a given susceptibility locus. Thus, peak allele sharing is expected to vary randomly around the locus, with some markers rising above—and other, nearby markers falling below—the criterion for significance (Kruglyak and Lander 1995). In complex phenotypes, true linkage peaks have been observed to shift over genetic distances as large as 20 cM (e.g., see Hall et al. 1990; Easton et al. 1993). Still, the question of whether the results in the new sample confirm linkage at the same location as was observed in the previous sample cannot be fully resolved by our data.

Our results are limited by the relatively small sample size. Small sample sizes could result in imprecise P values, especially in the SIBPAL analysis, which relies on asymptotic P values. This is particularly problematic in the parent-of-origin analyses, in which the range of effective sample sizes is 20–30 affected sib pairs. In order to increase sample size, we added seven families to the sample prior to genotyping. Although these families were recruited under less-stringent rules of ascertainment, they were clinically very similar to the other families, and excluding these families does not have a substantial impact on either the two-point or multipoint results.

The sample size may explain the failure to detect robust evidence of linkage to pericentromeric markers in this sample. Our simulations indicated that our sample of 30 pedigrees had good power to detect linkage, but the model under which our simulated data was generated may not fit the putative pericentromeric locus. Risch (1990) simulated the sample size needed to detect linkage to loci of varying relative risk, using a sample of affected sib pairs. On the basis of his results, our sample would have ~90% power to detect a locus conferring a relative risk $\geqslant 3$. The putative pericentromeric locus may confer a smaller relative risk in this particular sample and thus would not be detectable. It is also possible that our linkage finding at D18S548 (IBD = .56, $P = .0013$), ob-

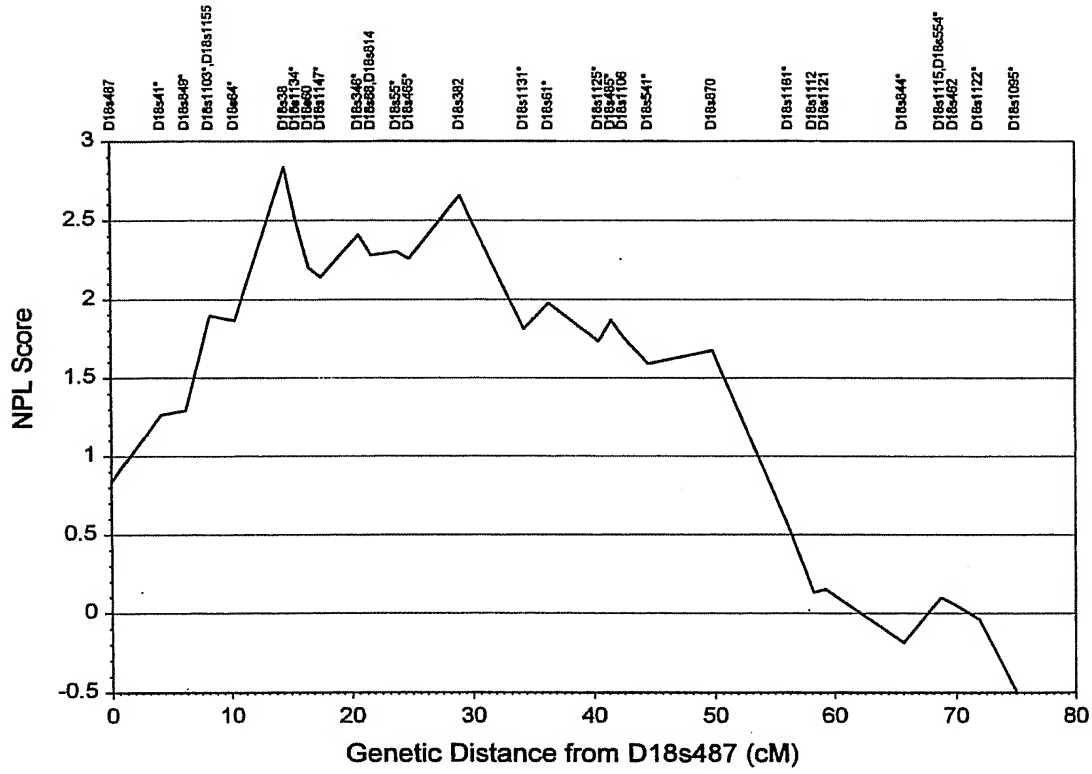

Figure 1 Results of multipoint nonparametric linkage analysis between BPAD and 31 markers on chromosome 18q in 58 families. Genetic distance (in cM) from the most centromeric marker, D18S487, is shown on the lower x-axis. Each marker used in the analysis is shown on the upper x-axis, according to map order and distance. Markers placed with $\geq$1,000:1 odds are denoted by an asterisk (*); all other markers are placed in their most likely location. The nonparametric linkage statistic given by the all-pairs option in GENEHUNTER (i.e., the NPL score) is shown on the y-axis.

served only when RUP sibs were included in the sample, reflects the same pericentromeric locus reported by Berrettini et al. (1994) and Stine et al. (1995). According to the Location Database Map (Collins et al. 1996), D18S548 is located <2 male cM and ~12 female cM qter from D18S56, which is the closest significantly linked marker in the Berrettini et al. (1994) report, and <1 male cM and ~4 female cM qter from D18S34, which yielded $P < .1$ in the Stine et al. (1995) report. This issue might be clarified by genotyping more markers in this region.

Previously we reported a parent-of-origin effect in BPAD. This was manifested clinically both by an increased rate of major affective disorder among maternal relatives and by a higher than expected proportion of families with no paternal transmission, consistent with imprinting or mitochondrial inheritance (McMahon et al. 1995). A parent-of-origin effect was also evident in the linkage between BPAD and chromosome 18 markers, in which most of the linkage evidence derived from families with an affected or apparently transmitting father (Stine et al. 1995). On 18q a parent-of-origin effect was also seen in the form of higher IBD scores and stronger linkage evidence for marker alleles on paternally transmitted chromosomes (Stine et al. 1995). Similar parent-of-origin effects have since been observed by groups studying linkage to the pericentromeric region of chromosome 18 in other family samples (Gershon et al. 1996; Nöthen et al. 1996). Gershon et al. (1996) reanalyzed the 22 families studied in the original report of linkage between BPAD and chromosome 18 (Berrettini et al. 1994) and concluded that there was a higher than expected proportion of families with no paternal transmission—and that the linkage evidence on chromosome 18 derived mostly from those families which did show apparent paternal transmission; the linkage results for paternally transmitted chromosomes were not reported.

A parent-of-origin effect was again seen in the present sample, but the effect was not consistently paternal (table 2). This unexpected finding may be the spurious result of small sample sizes. This finding may also reflect the complex genetics of BPAD, which make it impossible, on purely clinical grounds, to identify a transmitting parent. Although we relaxed the criteria for unilin-

eality for 3 of the 16 paternal families in the new sample, eliminating these families does not significantly change the results. It is also possible that detection of linkage to paternal and not to maternal chromosomes is influenced by the decreased male versus female recombination rates on chromosome 18q (Collins et al. 1996).

The site of linkage on 18q, first reported by Stine et al. (1995) and supported by this study, may or may not overlap with the putative 18q23 locus reported by another group (Freimer et al. 1996). Many of the same markers showed evidence of linkage in both samples (McInnes et al. 1996), and we cannot rule out the possibility that the same disease locus is being detected in both family samples. The Freimer et al. (1996) analysis was based only on subjects with BPI; our analysis, like those of Berrettini et al. (1994) and Stine et al. (1995), included cases of BPII and RUP. Genetic heterogeneity may be contributing to the difference in linkage localization. The low relative risk apparently conferred by this locus suggests that fine mapping will be difficult with linkage methods alone, unless much larger sample sizes are used (Kruglyak and Lander 1995).

We have here reported new evidence that BPAD is linked to chromosome 18q. These results strengthen the earlier evidence that a susceptibility gene for BPAD resides on the long arm of chromosome 18. Further studies are needed to clarify the nature of the observed parent-of-origin effect and to identify the inferred gene through positional cloning methods.

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Exhibit D

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PEDIATRIC RESEARCH

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A Deletion in the Long Arm of Chromosome 18 in a Child with Serum Carnosinase Deficiency[1]

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ABSTRACT

The dipeptides carnosine and anserine, found exclusively in meats, are hydrolyzed in serum by the enzyme carnosinase. Several reports of serum carnosinase deficiency describe a variable phenotype, which ranges from normal to severe psychomotor retardation, hypotonia, and myoclonic seizures in the first year of life. We report the case of a 30-mo-old girl with hypotonia, developmental delays, and tremor. Although consuming nominal quantities of meat, she excreted large amounts of carnosine and anserine. A strict meat-free diet ameliorated, but did not eliminate, these abnormalities. Serum carnosinase activity was found to be extremely low. Analysis of this child's chromosomes revealed a terminal deletion of chromosome 18 with breakpoint at q21.3. Neither parent exhibited this deletion, suggesting it was generated *de novo* in the patient or in a parental germ cell. Molecular studies showed that the patient's paternal chromosome 18 was deleted. Urinary carnosine excretion and serum carnosinase activity were normal in the patient's father. The mother had low carnosinase activity. The patient's brother exhibited moderate hypercarnosinuria and intermediate enzyme activity, consistent with the carrier state for carnosinase deficiency. Cumulatively, these findings suggest that the locus for this enzyme resides on the distal long arm of chromosome 18, and they are consistent with an unusual mechanism for the inheritance of this, typically autosomal recessive, condition. We conclude that this patient is likely hemizygous for the defect, having received the deficiency allele from her mother and, by virtue of the chromosomal deletion, no allele from her father. This represents the first report of a chromosomal abnormality in association with serum carnosinase deficiency and should aid in further localization of the gene encoding serum carnoosinase. (*Pediatr Res* **41: 210–213, 1997**)

Abbreviation

GABA, γ-aminobutyric acid

Carnosine (β-alanyl-L-histidine) is a dipeptide which is abundant in the skeletal muscle of most animals. Anserine (β-alanyl-1-methyl-L-histidine) is a component of skeletal muscle in birds, but is absent from human tissue (1, 2). Both of these dipeptides are hydrolyzed by an enzyme present in serum, carnosinase (EC 3.4.13.3).

Carnosinase exists in two isoforms (3). One form (tissue carnosinase; M_r = 90 000) is present in various tissues including liver, kidney, and spleen, but not skeletal muscle. The other form (serum carnosinase; M_r = 155 000) is found primarily in serum, but also in brain and spinal fluid (4). These enzymes differ not only in their distribution and molecular weight, but also in specificity. One very important difference is that serum carnosinase is able to hydrolyze homocarnosine (GABA-L-histidine), whereas tissue carnosinase is not. Consequently, profound elevations in cerebrospinal fluid homocarnosine have been found in children with serum carnosinase deficiency (5).

Deficiency of serum carnosinase has been described in several sibships in conjunction with tremor, myoclonic seizures, hypotonia, and profound psychomotor retardation (6–10). The clinical presentation of this disorder is quite variable, however, and there exists at least one detailed report of low enzyme activity in an apparently normal sibling of an index case (11). We describe the case of a 30-mo-old white female patient referred for evaluation of developmental delays with hypotonia and a tremor. The coexistence of this rare enzymatic deficiency and a gross deletion on the long arm of chromosome 18 suggests that the gene for serum carnosinase resides in this portion of the genome.

METHODS

Case report. P.E.Z. is a 30-mo-old white girl with tremor, hypotonia, and global developmental delays. She was born at term by vaginal delivery and weighed 6 pounds and 10 ounces. Her development appeared normal until 6 mo of age, but began to plateau by 12 mo. At that time, she developed hypotonia and

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[1] These data were presented, in part, at the Xth Annual Meeting of the Society for Inherited Metabolic Diseases.

tremor and was found to have a urinary tract infection. The family's diet is strictly lacto-ovo-vegetarian, and the child had not consumed any meats until this time. The family introduced meats into her diet in hopes of improving her symptoms and growth. Shortly thereafter, she developed a tremor, which worsened for approximately 3–4 mo but seemed to improve after the elimination of meats from her diet. Her development has improved, but has not returned to normal for age. Physical examination reveals her height, weight, and head circumference are at the fifth percentile. A neurologic examination was notable for a tremor (accentuated by intent), ataxia, and mild generalized hypotonia.

Magnetic resonance imaging revealed normal brain anatomy. Blood chemical analyses and hematologic profiles were essentially normal. Extensive metabolic testing demonstrated no abnormalities except for quantitative urine amino acids that revealed striking hypercarnosinuria and anserinuria. As these data suggested the diagnosis of serum carnosinase deficiency, this child has been maintained on a low carnosine diet with little change in her clinical condition. Urinary carnosine has remained modestly elevated in the absence of detectable hypercarnosinemia.

Biochemical analysis. Twenty-four-hour urine collections from the patient, family members, and 18 pediatric control subjects were assayed for amino acids and creatinine using standard techniques (12). Serum carnosine quantitation was performed using a Beckman 6300 high performance amino acid analyzer. Serum carnosinase activity was assayed in the proband's family, as well as 69 normal control subjects varying in age from 6 mo to 44 y. The stability of serum carnosinase is not well studied, but it has been found to be stable for several weeks, if samples are kept frozen (13). For this reason, serum samples were frozen and stored at −70°C until the morning of assay. Interassay reliability for frozen samples assayed 2 mo apart was 6.7%. In contrast, carnosinase activity decreased by an average of 16.8% when samples were stored for a period of 14 d at 4°C before assay.

The serum carnosinase assay was a modification of the method of Lenny *et al.* (13). Twenty-five microliters of serum were added to 0.15 mL of a solution containing 2.5 mmol/L $CdCl_2$ and 15 mmol/L sodium citrate. To this 0.225 mL of 125 mmol/L NH_4OH-HCl buffer (pH 8.5) was added. After 10 min at 30°C, 0.10 mL of 100 mmol/L carnosine in NH_4OH-HCl buffer (pH 8.5) was added, and the reaction was allowed to proceed for 20 min at 30°C. The reaction was terminated with 0.50 mL of 0.6 N trichloroacetic acid, and insoluble material was precipitated by centrifugation at 800 × *g* for 10 min. Histidine content was measured in 0.50 mL of the trichloroacetic acid supernatant using the method of Ambrose *et al.* (14). The sensitivity of our measurements was increased 5-fold over existing techniques (13), using a very sensitive fluorescence spectrophotometer (Perkin-Elmer LS-5).

Cytogenetic analysis. Peripheral blood was obtained for chromosome analysis on P.E.Z. and her parents. Lymphocytes were cultured at 37°C for 72 h in tissue culture medium RPMI 1640 with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer and 20% FCS. Ethidium bromide was used to achieve chromosome elongation (15). Cells were harvested, and slides were prepared and aged by standard methods. Chromosome morphology was examined by G-banding.

Molecular analysis. Microsatellite markers were amplified by the polymerase chain reaction. Each reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 40 ng of each primer, 100 μM dATP, dGTP, and dTTP, 2.5 μM unlabeled dCTP, 16.7 nM ^{32}P-labeled dCTP (3,000 Ci/mmol), and 0.45 U of Amplitaq DNA polymerase (Perkin-Elmer), in a total volume of 15 μL. Samples were denatured at 94°C for 5 min, then 27 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min were finally followed by a 6-min final extension period at 72°C. Polymerase chain reaction products were electrophoresed on a 6% acrylamide/7 M urea gel and visualized by autoradiography with XAR (Kodak) film.

RESULTS

Quantitative amino acid analysis of the patient's urine revealed striking hypercarnosinuria and anserinuria (Table 1). When all dietary sources of meat were eliminated, hypercarnosinuria persisted. The introduction of white poultry meat into the diet, for just 48 h, was accompanied by a marked elevation in anserine, in the presence of low 1-methylhistidine (a metabolite derived primarily from serum carnosinase action upon anserine). The patient's parents did not exhibit hypercarnosinuria on a meat-free diet. However, urine amino acid analysis of the patient's brother demonstrated moderately increased carnosine excretion on a vegetarian diet.

Fifteen chromosome spreads were analyzed from G-banded preparations and revealed a terminal deletion involving the long arm of chromosome 18 with most likely breakpoint 18q21.3 (Fig. 1). The karyotype was designated 46,XX, del(18)(q21.3). Parental karyotypes were normal.

The breakpoint of the deletion was determined to lie within an 8-centiMorgan region, between the dinucleotide repeat markers

Table 1. *Urinary excretion of carnosine, anserine, and 1-methylhistidine in 24-h urine collections from proband on various dietary regimens*

Urinary excretion (mmol/mol creatinine)	Normal diet		Meat-free diet					Poultry diet	
	Proband	Controls (mean ± SD)	Proband	Sibling	Mother	Father	Normal* (mean ± SD)	Proband	Normal†
Carnosine	932	23.3 ± 8.9	113.2	14.0	—	—	1.1 ± 0.5	686	
Anserine	467	16.4 ± 10.9	—	—	—	—	0	2979	
1-Methylhistidine	17	16.0 ± 10.2	1.2	—	—	0.9	13.6 ± 9.0	62	

Family members were studied on a meat-free diet only. Control data were generated from 18 pediatric patients on *ad libitum* feedings.

* Reference values from 12 patients [from Lunde *et al.* (10)].

† Reference range not clearly established. Available data suggests a molar excess of 3.2–4.9:1 for 1-methylhistidine:anserine [from Perry *et al.* (6)].

— = none detected.

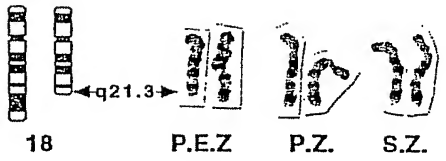

Figure 1. High resolution cytogenetic analysis of chromosome 18 in the proband and her parents. The deletion consists of all material distal to 18q21.3.

D18S38 and D18S42 (16). The patient had two distinct alleles, and was therefore not deleted, for the D18S38 marker in 18q12.3 (left panel, Fig. 2), as well as the more proximal 18q markers D18S35, D18S34, and the short arm markers D18S40 and D18S59 (data not shown). These chromosome 18 markers, which displayed biparental inheritance, were all consistent with the presumed paternity of this patient. The patient had one allele at the D18S42 marker in 18q22.1, corresponding to one of the two maternal alleles seen for this marker (right panel, Fig. 2). There was no evidence of a paternal contribution at this marker, or the more distal 18q markers D18S43, MBP, and D18S70 (data not shown). These studies reveal that the deletion was present on the paternal chromosome 18. Paternity was further investigated by examining highly polymorphic markers on chromosomes 11, 13, 16, and 22. The patterns observed were consistent with paternity for all of these markers as well.

Carnosinase activity was detectable throughout a wide range of activities. Normal control values varied from 0.42 to 40.5 μmol/mL/h. Serum carnosinase activity in adults (23.2 ± 10.6 μmol/mL/h; mean ± SD) is somewhat variable (Fig. 3). Enzyme activity in the proband's father (19.4 ± 0.36 μmol/mL/h) is in the middle of the adult normal range. Moreover, the mother's carnosinase activity (3.7 ± 0.24 μmol/mL/h) is in the lowest fifth percentile, consistent with the carrier state for this deficiency. A log plot of carnosinase activity *versus* age (Fig. 4) demonstrates that the activity of this enzyme increases with age throughout childhood, approaching adult normal values at about age 14 y. Calculation of a prediction interval for enzyme activity in children reveals that the proband and, to a lesser extent, her unaffected sibling lie outside of values which would be predicted with 95% confidence. The proband's carnosinase activity (0.16 ± 0.07 μmol/mL/h) is much lower than similar aged and even younger control subjects. Of note, the brother's activity (0.65 ± 0.10 μmol/mL/h) is comparable with values previously considered to be consistent with carnosinase deficiency (11).

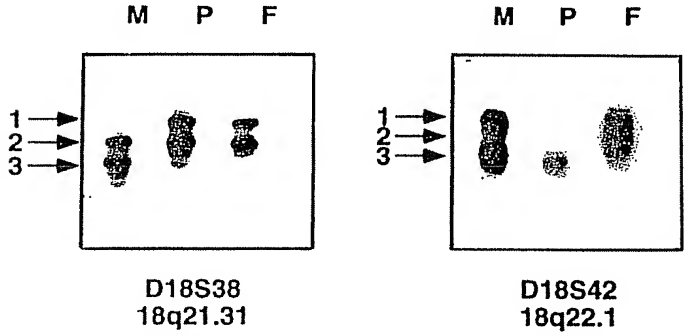

Figure 2. Autoradiographs from the patient (*P*), her mother (*M*), and her father (*F*) using nucleotide repeat markers D18S38 (left) and D18S42 (right). For D18S38, the patient has inherited allele 1 from her father and allele 2 from her mother, suggesting that the allele breakpoint is distal to this marker at 18q21.31. In the case of D18S42, the patient has inherited allele 3 from her mother but has no paternal allele, indicating a deletion on the paternal chromosome 18, which includes this portion of 18q22.1.

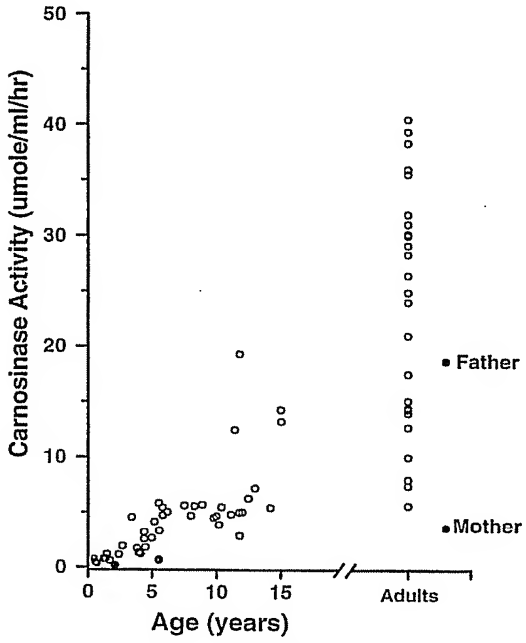

Figure 3. Serum carnosinase activity plotted against age in 69 controls (○) as well as the proband's family (●).

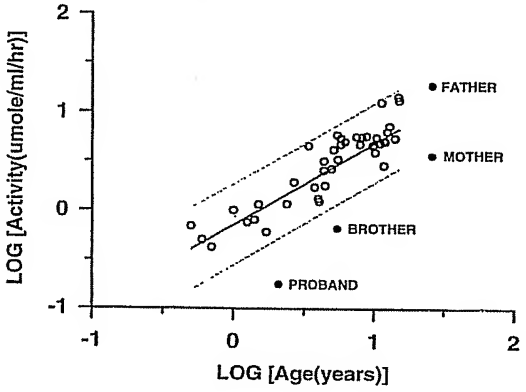

Figure 4. A log/log plot of serum carnosinase activity *vs* age clearly differentiates the proband from normal controls at all ages tested. The thin line represents the 95% prediction interval based upon this normative sample. Mildly reduced activity in the patient's brother suggests that he is heterozygous for carnosinase deficiency.

DISCUSSION

We report the case of a 2.5-y-old child with serum carnosinase deficiency and a concomitant deletion in the terminal portion of the long arm of chromosome 18. To our knowledge, this is the first report of a chromosomal abnormality in association with this metabolic disorder. The presence of normal carnosinase activity and normal urinary carnosine excretion in the patient's father suggests he does not harbor a defective gene for this rare autosomal recessive disorder. Unfortunately, carnosinase activity in adults demonstrates considerable variability, making carrier de-

tection difficult. However, the presence of low/normal carnosinase activity in the patient's mother would be consistent with the carrier state. Moreover, the moderate hypercarnosinuria and low carnosinase activity exhibited by the patient's brother suggest that the carrier state for this condition presents a normal phenotype despite hypercarnosinuria.

These findings are consistent with a unique genetic phenomenon leading to autosomal recessive inheritance of this disorder. We postulate that this child inherited a mutant allele from her mother and that this defect remained unbalanced by a normal paternal allele due to the gross chromosomal deletion in the long arm of chromosome 18. In favor of this assertion is the demonstration through molecular analysis that indeed the 18 q- is of paternal lineage. Furthermore, molecular analysis clearly established the paternity of this child. These data, cumulatively, suggest that the gene encoding this enzyme likely resides in this region of chromosome 18. Potential alternative explanations for these data include the rare possibilities of uniparental disomy or spontaneous mutation of the carnosinase gene in the paternal germ cell. As the paternal germ line has clearly been demonstrated to be the origin of the overt chromosomal deletion, it seems unlikely to postulate a spontaneous mutation in addition to this deletion.

Lenny *et al.* (13) previously reported a sensitive fluorometric assay for serum carnosinase activity, which takes advantage of the fact that cadmium is an activator of this enzyme *in vitro*. By altering the incubation conditions and using a very sensitive fluorescence spectrophotometer, we were able to improve on the sensitivity of this assay by approximately 5-fold. This led to reliable segregation of the proband from similarly aged, and even younger, normal children.

The significance of the improved sensitivity of this assay is magnified by a number of reports of "low" carnosinase activities in unaffected family members of index cases (8, 11). Such reports have led to the suggestion that clinical manifestations do not necessarily accompany the biochemical phenotype. We suspect that a number of carriers for this disorder were erroneously labeled as deficient, due to the inability of previous methods to clearly differentiate heterozygotes from homozygotes. Careful analysis of these reports reveals that the subjects, in fact, had only mildly reduced levels of carnosinase activity and were labeled as deficient primarily due to the presence of hypercarnosinuria. Moreover, the degree of hypercarnosinuria evident in these subjects was considerably milder than that demonstrated in our patient and the preponderance of previously reported cases.

In the present case study, a mild degree of hypercarnosinuria was evident in the patient's phenotypically normal brother. Although hypercarnosinuria on a meat-free diet was not demonstrable in the patient's mother, both brother and mother had relatively low carnosinase activity. These facts highlight the hazards of interpretation of urinary carnosine excretion and the importance of a sensitive serum assay in the definitive diagnosis of serum carnosinase deficiency. Unfortunately, normal adult carnosinase activity demonstrates considerable variability, making carrier detection difficult even with this improved sensitivity. As a result, we cannot completely rule out the possibility that the mother's and brother's marginal carnosinase activity are statistical coincidence. This possibility seems unlikely in light of the brother's hypercarnosinuria. In any case, the absence of carrier status in these individuals would not alter the basic premise, which is that the gene for serum carnosinase resides on the long arm of chromosome 18.

The presence of neurologic dysfunction in this child, and others with serum carnosinase deficiency, gains potential significance in light of the recent identification of high levels of this enzyme in the brains of higher primates (4). Interestingly, this enzyme is absent from the brains of lower primates and most other mammals. As serum carnosinase is essential to the conversion of homocarnosine to GABA and histidine, it is likely that one of this enzyme's most vital functions is in providing a source of the neurotransmitter GABA in the CNS. The fact that homocarnosine is found in high concentration (>1 mM), with a distinct pattern of localization in the CNS (17), lends further credence to this function. We postulate that homocarnosine may represent a reservoir of GABA which is unavailable to carnosinase-deficient patients, and that hypercarnosinuria merely serves as a marker for this profound neurologic disorder.

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Exhibit E

NEUROLOGICAL DISEASE IN A CHILD WITH CARNOSINASE DEFICIENCY

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Wisniewski, K., Fleisher, L., Rassin, D. and *Lassmann, H.: Neurological disease in a child with carnosinase deficiency.* Neuropediatrics 12: 143–151 (1981). Carnosinase deficiency presented as a progressive neurological problem with sensory polyneuropathy in a 12-year-old male. Carnosinuria was present, even on a meat-free diet, although carnosinemia was not observed. An increased amount of unmetabolized carnosine was found in the urine after a carnosine load. Serum and tissue (liver and nerve) from the patient showed deficient carnosinase activity (*Fleisher* et al. 1978, 1980).

Morphometric and fine structural studies on the nerve and skin biopsies are presented, as is a review of the literature on carnosinase deficiency in mentally retarded patients.

Carnosinase deficiency polyneuropathy

Introduction

Deficient activity of the enzyme carnosinase causes impaired cleavage of carnosine to β-alanine and histidine. This rare metabolic disorder was first described by *Perry* et al. (1967, 1968). Carnosine and its homologues were observed in rat brain by *Hosein* and *Smart* in 1969, and were subsequently found in the CNS of other mammalian species, including man (*Perry* et al. 1968, *Pisano* et al. 1961, *Shaw* and *Heine* 1965). The highest concentration of carnosine in the CNS is found in the olfactory bulb (*Morgolis* and *Grillo* 1977, *Neidle* and *Kandera* 1974).

Our patient with carnosinase deficiency (*S. T.*) presented with progressive neurological problems and peripheral sensory neuropathy. The biochemical and enzymatic studies of this case are reported in separate publications (*Fleisher* et al. 1978, 1980).

Case report

S. T., a 12-year-old white male, was the product of a second pregnancy of 36 weeks gestation, which was complicated by incomplete placenta praevia with bleeding on the day of delivery. The delivery and the perinatal and postnatal stages were uneventful. Birth weight was 7 lbs.

Medical history

In early infancy episodes of opisthotonic positioning, arching of the back and occasionally, jerky myoclonic movements were noted. *S. T.* smiled at

1 month, had head control at 3–4 months, rolled over from prone to supine at 4–5 months, and started babbling between 7 months and 1 year of age. He reached spontaneously for objects at 4–5 months of age. At the age of 5–6 months the spontaneous reflex to reach for objects or turn over was completely lost and spasticity was noted. He has been on phenobarbital periodically since 9 months of age. At the age of 1 year he lost the ability to babble and began drooling frequently. In the second year of life, athetoid movements were observed. At the age of 3–4 years a few episodes of grand mal seizures were noted. The last seizure was observed at the age of 4. In the 4th–5th year distal muscle atrophy was seen. At the age of 6 years he regained the ability to turn over, and at 8 years he regained the ability to sit. At 10 years he developed difficulty in swallowing.

Family history

Both parents are college graduates. The mother is Italian and the father is of Ukranian and Irish origin. *S. T.* has two healthy brothers. There is no history of consanguinity or central nervous system problems on either the maternal or paternal side.

General examination

His general appearance is normal except for flattening of the occiput and prominence of both parietal regions with some asymmetry (the right greater than the left). His face is normal except for the upper lip which is shaped like an inverted "U", and a high arched palate. Mild kyphoscoliosis is apparent when the child is sitting. His fingers and toes are unusually long. The second toe overrides the third which is in flexion, and the fourth and fifth toes show clinodactyly. His height and weigth are in the 50th percentile and his head circumference is in the third percentile.

Neurological examination

S. T. is alert and has good eye contact. He is mute and makes no attempt to communicate. Cranial nerves II–XII are grossly intact although vision and hearing cannot be well determined. The child responds occasionally to auditory and visual stimuli. The optic discs are pale. The muscle tone is increased in all extremities. Marked fixed contracture in the hip, knee and ankle and mild fixed contracture in the shoulder, ulnar and interpharyngeal joints were noted. Muscle strength could not be determined. Muscle bulk is good except for distal wasting of the small muscles of the hand, mainly the thenar and hypothenar muscles. The child has bilateral cortical thumbs. His reflexes are symmetrical 3 $^{+}$, bilateral extensor plantar responses. Jaw and snout reflexes are present and abdominal reflexes are absent. Involuntary movements of the athetoid type are seen in both upper extremities. He reacts to painful stimuli. No lacrimation is seen when crying. No dysmetria or intension tremor is apparent when the child reaches for objects.

Laboratory findings

Skull X-ray, chest X-ray, bone survey, SMA_{12}, VDRL, CSF, urinalysis, CBC and chromosomal analysis were all found to be normal. A CAT scan showed cortical atrophy with a mild degree of ventricular dilation. An EEG at the age of 2 showed dysrhythmia grade 2 and a multifocal spike, and at the age of 10 showed dysrhythmia grade 3. Carnosinuria was detected by urinary amino acid analysis, even when the patient was on a meat-free diet. However, no carnosinemia was observed. An increase in unmetabolized carnosine in the urine was observed after a loading test with carnosine. Serum and tissues (liver and nerve) had deficient carnosinase activity (*Fleisher* et al. 1978, 1980). Electrophysiological studies showed and absence of sensory evoked responses and a decreased amplitude of evoked muscle desponses in the right median, ulnar, peroneal and tibial nerves. Nerve conduction studies and EMG were normal.

Materials and methods

Sural nerve and skin biopsies were fixed in 3 % glutaraldehyde buffered with 0.1 M phosphate buffer, pH 7.4 for 2 hours. The specimens were dissected and embedded, partially in paraffin for light microscopy and partially, after postfixation, in phosphate buffered OsO_4 in Epon for electron microscopy. Light microscopic stains included HE, *Luxol* fast blue, *Cresyl* violet and *Bodian* on paraffin sections and *Toluidine* blue on *Epon* sections. Electron microscopy was performed on thin sections stained with uranyl acetate and lead citrate, using a *Phillips* EM 300 microscope. Histograms of the size distribution of myelinated and unmyelinated fibers in the sural nerve biopsy were performed according to the method of *Dyck* et al. (1971). Myelinated fibers were counted from photomicrographs (magnification X 100) of two nerve fascicles, containing a total number of 1,513 myelinated axons. After determination of the square dimensions of the fascicles, the number of nerve fibers was recalculated on the basis of square mm fascicle area. A total number of 915 unmyelinated fibers were counted from randomly sampled electron micrographs of four sural nerve fascicles at a magnification of X 10,000, and calculated on the basis of square mm fascicle area. As the density of unmyelinated nerve fibers in different fascicle areas was variable, the counted values were corrected for the whole nerve cross section. For the purpose of quantitative determinations, the area of the electron micrographs were relocated on corresponding light micrographs, and the square dimension of unmyelinated fibers was determined with a morphometric raster. The obtained values were compared with the square dimension of unmyelinated nerve fibers within the whole biopsy specimen and the counted values were then recalculated. The correction procedure resulted in a 5 % reduction of the number of total fibers/mm^2.

Table I Published patients with carnosinase deficiency

Author	Year	Sex	Age	Age at onset	Seizure	Mental retard.	Pyram. signs	Ocular abnorm.	Muscle wasting
Perry	1967	M	3y	3mo	+	+	+	+	?
et al.		M	8mo	2mo	+	+	+	+	?
Heswijk et al.	1969	M	4y	6mo	+	+	+	+	?
Terplan et al. and	1972	M	7.5y	6mo	+	+	+	+	+
Murphy	1973	M	4y	2mo	+	+	+	+	?
et al.		F	6y	0	0	0	0	0	0
Gordon et al.	1977	M	1.5y	0	0	0	0	0	0
Dolman	1978	M	13y	6mo	+	+	+	0	0
Ours		M	12y	5mo	+	+	+	+	+

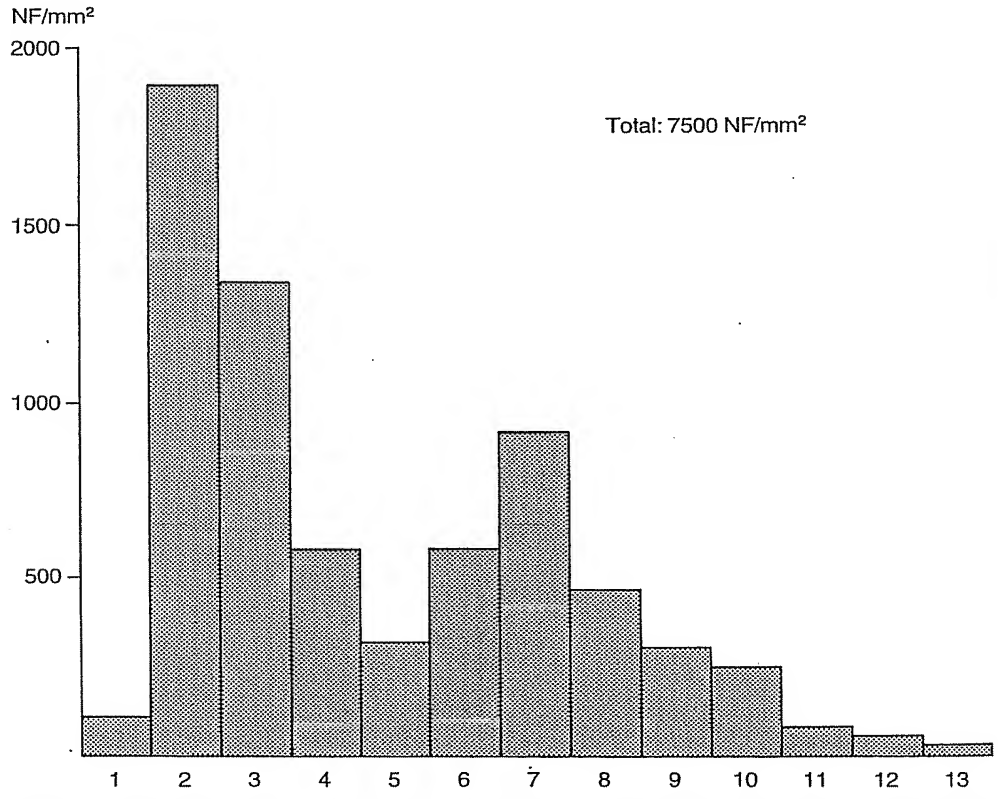

Table II Histogram of size distribution of myelinated nerve fibers in the nerve sural biopsy. NF: Nerve fibers

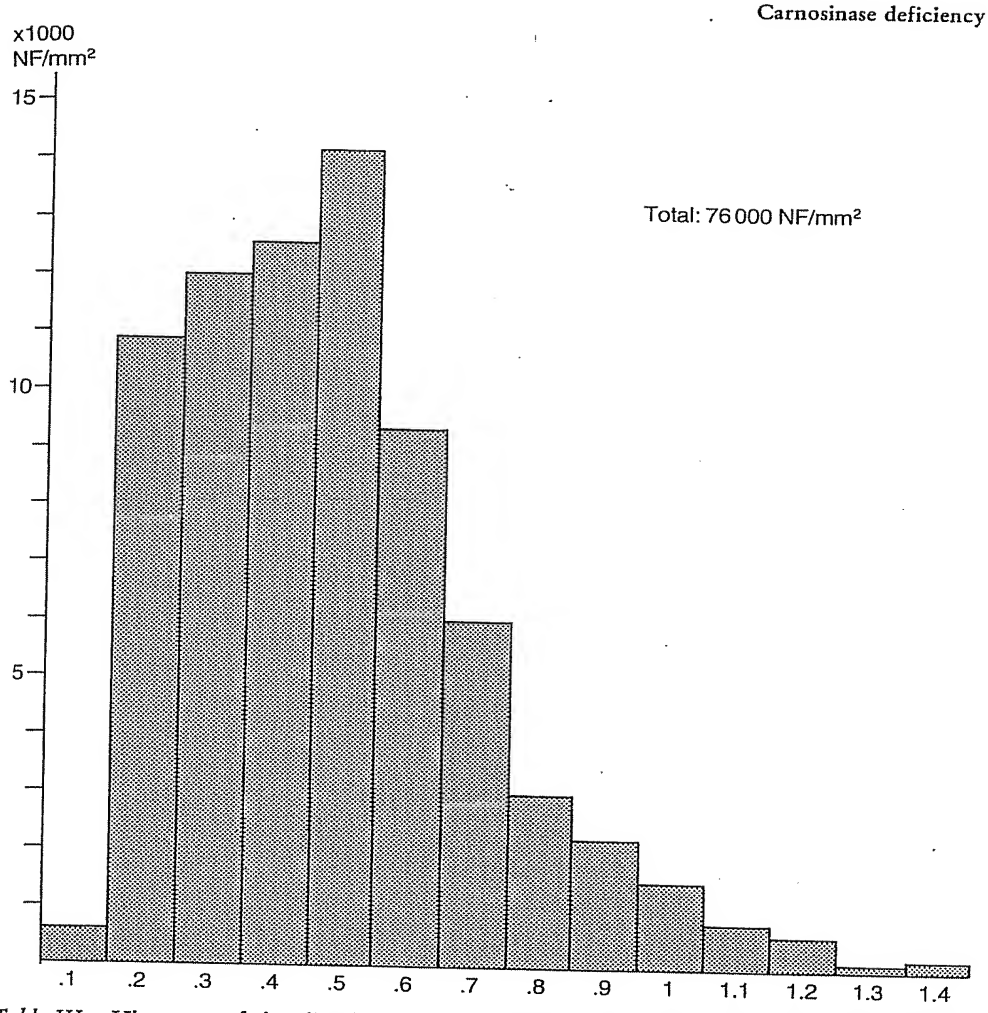

Table III Histogram of size distribution of unmyelinated nerve fibers in the sural nerve biopsy

Results

Sural nerve

Pathological alterations in the sural nerves were not seen under light microscopy. Specifically, no spheroids, giant axons, demyelination or myelin degradation products were present in cross- or longitudinal-sections of the biopsy. The histogram of the size distribution of myelinated nerve fibers was normal (Table II).

Myelinated nerve fibers, as seen under the electron microscope, were minimal. A few axons showed an unusual accumulation of glycogen within the axoplasm. The unmyelinated axons were increased in number (corrected values: 76,000 F/mm², Table III). Their size distribution was unimodal

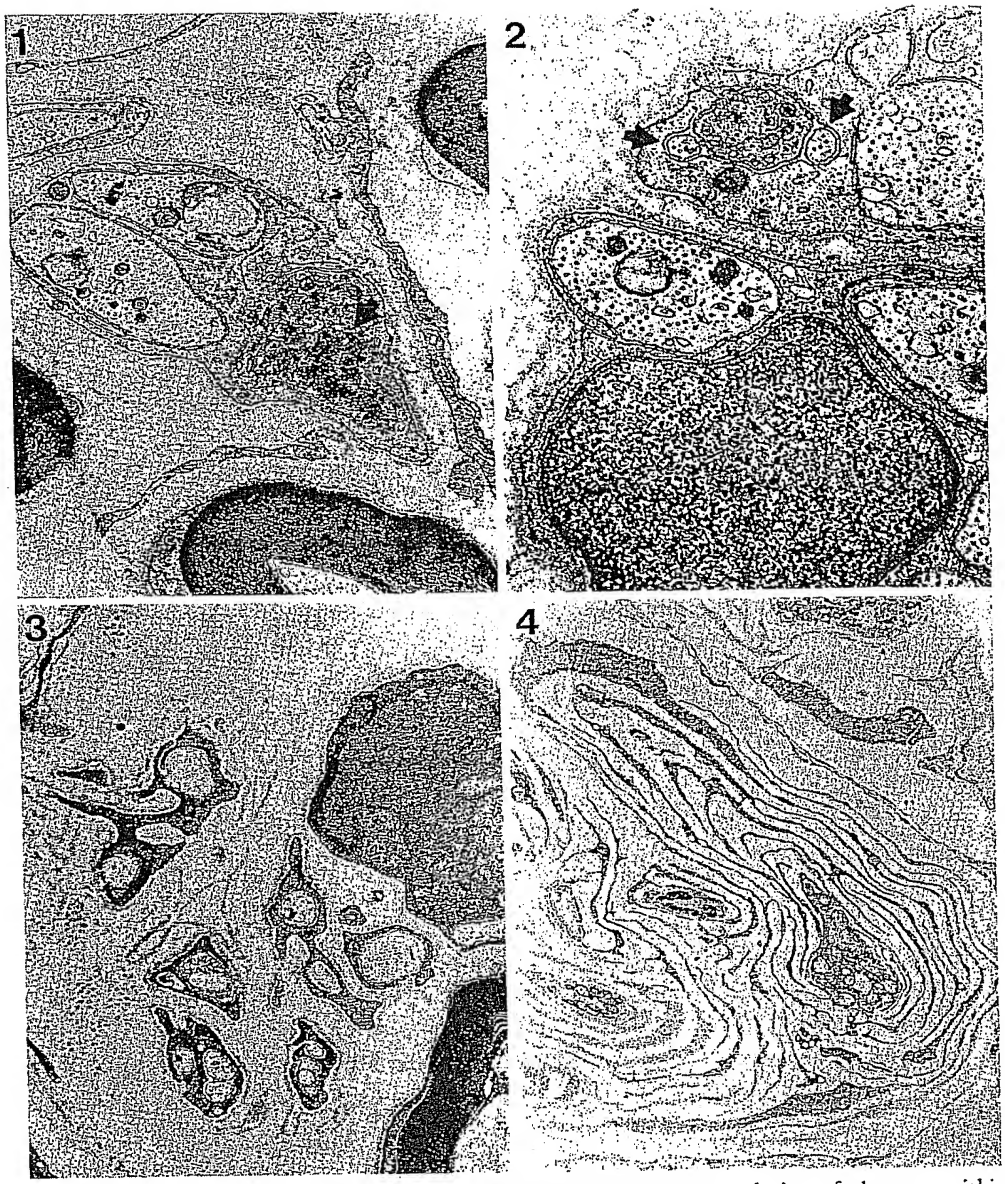

Fig. 1 Sural nerve; unmyelinated nerve fiber; One axon shows accumulation of glycogen within the axoplasm (arrow). X 18200

Fig. 2 Sural nerve; unmyelinated nerve fiber; One axon with increased density of the axoplasm, in close vicinity two small axonal profiles (arrow) probably representing regenerating sprouts. X 14600

Fig. 3 Skin, nerve fascicle from the deeper layers of the cutis; unmyelinated nerve fibers with axonal rarefication, ramifying *Schwann* cell processses and basement membrane duplications. X 11000

Fig. 4 Skin; nerve fascicle from the deeper layers of the cutis; Several axons with increased organelles and fibrillar material in the axoplasm, surrounded by multiple layers of slender ramifying cell processes. X 5500

with a high proportion of small unmyelinated fibers having a diameter of 0.2–0.4 u (Table III). Some axonal profiles contained large amounts of glycogen (Fig. 1), others showed increased electron density within the axoplasm (Fig. 2). In addition, in the vicinity of these fibers, very small axonal profiles (0.2–0.3 u diameter, Fig. 2) were often found. This probably indicates axonal regeneration (*Ochoa* and *Mair* 1969).

Skin

The histological organization of epidermal structures, skin appendages and connective tissue layers were all normal. Cutaneous nerve fibers and terminals frequently showed rarification of nerve fibers and endoneural fibrosis. Under electron microscopy, unmyelinated nerve fibers were seen to be predominately abnormal. These fibers showed rarification of axonal structures with formation of collagen pockets, ramification of *Schwann* cell processes, and basement membrane duplications (Fig. 3).

In one small fascicle in the deeper layers of the cutis there was increased cellular density with concentric arrangement of cell processes (Fig. 4). This structure included several axons containing increased amounts of organelles and fibrillar material, surrounded by concentrically arranged ramifying cell processes. The slender cellular profiles were surrounded by a partially discontinuous basement membrane comprising mainly fibrillar material. Few mitochondria and surface vesicles were seen, with no cellular contacts between the cell processes (Fig. 4).

Discussion

In the last ten years, eight cases have been reported with deficient serum carnosinase activity (*Dolman* 1978, *Gordon* et al. 1977, *Murphy* et al. 1973, *Perry* et al. 1967, *Perry* et al. 1968, *Terplan* and *Cares* 1972, *van Heesuijk* et al. 1969). All patients reported were boys with similar clinical symptomology with the exception of one 6-year-old female sibling described by *Murphy* et al. (1973) and a 1.5-year-old male reported by *Gordon* et al. (1977) with no clinical signs. The oldest reported patient was 13 years of age (*Dolman* 1978). The age of onset is generally 3–6 months after birth, and the symptoms include seizures, progressive pyramidal, extrapyramidal and suprabulbar signs, with or without occular abnormalities, and peripheral neuropathy. The clinical picture indicates diffuse CNS dysfunction.

In a light microscopic neuropathological study of a similar case, *Terplan* et al. (1972) described muscular atrophy and severe axonal degeneration and demyelination in the peripheral nervous system. They also described muscle wasting, but nerve conduction studies and EMG were not performed. In the CNS, demyelination and spheroids in the pyramidal and spinocerebellar tracts were present, along with neuronal loss in the cerebellum,

nucleus gracilis, nucleus cuneatus and in the grey matter of the spinal cord. *Dolman* (1978) reported a similar case of carnosinase deficiency with neuronal loss in the brain and brain stem, and sponginess or cavitation in the putamen. However, light microscopy of the spinal cord, peripheral nerves and muscles showed no changes. In our case no changes were seen using light microscopy. With EM the most striking pathological feature was the unusual accumulation of glycogen and the increased axonal density, mainly in some of the unmyelinated nerve fibers, together with the increase of small unmyelinated axonal profiles. These findings may be interpreted as a mild degenerative and regenerative process, predominantly involving unmyelinated nerve fibers. Compared with the sural nerve in cutaneous nerve fascicles and terminals, the pathological alterations appear to be more pronounced. However, it should be remembered that a larger degree of abnormalities may be found in these nerves in normal individuals (*Stockinger* et al. 1977).

The different pathological alterations observed by *Terplan* et al. (1972), *Dolman* (1978), and in our case raise the question of whether carnosinase deficiency is causally related to specific pathological changes. Until additional cases become available, we cannot determine whether carnosinase deficiency actually results in pathological disease entities, or, alternately, whether the pathological alterations found in our case are due to other metabolic, nutritional or local factors unrelated to carnosinase deficiency.

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Exhibit F

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Homocarnosinosis: lack of serum carnosinase is the defect probably responsible for elevated brain and CSF homocarnosine

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Summary

Patients afflicted with homocarnosinosis have elevated concentrations of homocarnosine in brain and CSF. It has been reported that they lack brain homocarnosinase. However, we have found that these patients are deficient in serum carnosinase, a dipeptidase which hydrolyzes homocarnosine about 5% as rapidly as it splits carnosine. Homocarnosinase could not be detected in normal human brain extracts after isoelectric focusing or DEAE-cellulose column chromatography. The ability of brain extracts to hydrolyze homocarnosine thus appears to be attributable solely to the serum carnosinase which is present because of serum trapped in the brain sample. Preliminary data indicate that homocarnosinase is probably not present in 13 other human tissues. Normal CSF contained serum carnosinase, whereas the CSF of a homocarnosinosis patient was lacking this enzyme. Thus it appears that the elevated concentrations of homocarnosine in the CSF of homocarnosinosis patients are attributable to serum carnosinase deficiency.

Introduction

Homocarnosinosis is a rare metabolic disorder which has been reported in three members of one Norwegian family by Gjessing and Sjaastad [1] and Sjaastad et al [2,3]. These patients have CSF homocarnosine concentrations which are 20 times normal [1] and are afflicted with progressive mental deterioration, spastic paraplegia and retinal pigmentation.

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Enzymes: Carnosinase (EC 3.4.13.3); homocarnosinase (EC 3.4.13.13); aldolase (EC 4.1.2.13).

A hog kidney dipeptidase (homocarnosinase) which hydrolyzes homocarnosine was described by Lenney et al [4]. This enzyme was present in rat uterus, kidney, and liver but was not detectable in rat serum or brain using a sensitive fluorometric assay [4]. Kish et al [5] reported that homocarnosinase was present in human brain. Perry et al [6] analyzed a frontal cortex biopsy sample from one of the homocarnosinosis patients and found that it contained four times the normal concentration of homocarnosine, but was lacking homocarnosinase. The high concentrations of cortex and CSF homocarnosine were attributed to the deficiency in brain homocarnosinase.

In 1982, Lunde and coworkers [7] studied the compounds excreted by the homocarnosinosis patients when they consumed a meal high in anserine and carnosine. As compared with normal controls, the patients excreted higher levels of carnosine and anserine and lower concentrations of histidine and 1-methyl histidine.

Lenney et al [8] found that human serum carnosinase differed from tissue carnosinase. The circulating enzyme hydrolyzed homocarnosine whereas the tissue dipeptidase did not.

In this communication, we show that the homocarnosinosis patients are deficient in serum carnosinase. Furthermore, the enzyme homocarnosinase was not detectable in samples of normal human cerebral cortex. The hydrolysis of homocarnosine by extracts of brain was attributed to the activity of serum carnosinase which was trapped within the blood vessels of brain samples. Serum carnosinase was also found to be present in normal CSF.

Materials and methods

Materials

Serum samples from the three homocarnosinosis patients, a CSF sample from one of these patients, and serum samples from three normal volunteers were shipped by air on dry ice from Norway to Honolulu, where they were analyzed. A model 200 ultrafiltration cell and Diaflo YM30 ultrafiltration membranes were from Amicon (Danvers, MA, USA). Homocarnosine was from Aldrich (Milwaukee, WI, USA), DEAE-cellulose from Bio-Rad Labs (Richmond, CA, USA), and charcoal (Norit A) from J.T. Baker, Philipsburg, NJ, USA. Disc gel electrophoresis chemicals were from Eastman Kodak (Rochester, NY, USA), and ampholytes were from Isolab (Akron, OH, USA). Carnosine, Sephadex, aldolase, bovine serum albumin, ovalbumin, and all other chemicals were from Sigma Chem. Co., St. Louis, MO, USA.

Enzyme assays

Serum carnosinase was measured using a published procedure [8] in which the free histidine liberated from carnosine was estimated fluorometrically after reaction with *o*-phthalaldehyde.

Tissue carnosinase was assayed by the same procedure except that the digest contained 20 mmol/l carnosine, 0.2 mmol/l $MnCl_2$, 1.6 mmol/l dithiothreitol, and 50 mmol/l sarcosine-HCl buffer, pH 9.5.

In measuring homocarnosine-splitting activity, the digest contained 4 mmol/l homocarnosine, 0.4 mmol/l $CoSO_4$, and 50 mmol/l Tris-HCl buffer, pH 7.6.

Sensitivity was increased by the use of a micro modification of the histidine assay. To 0.2 ml of the trichloroacetic acid supernatant were added 0.3 ml 1 mol/l NaOH, 0.2 ml 0.2% *o*-phthalaldehyde in 95% ethanol, and 0.2 ml 4 mol/l H_3PO_4. Other details of the procedure were as described for the serum carnosinase method [8].

Normal human tissues for the homocarnosine-splitting assay were obtained at autopsy, which was performed 5–20 h after death. The organs or tissue samples were placed on ice for about 1 h and then they were frozen at −70°C. Excess blood had drained off during the period before freezing. To 5 g of frozen tissue was added 25 ml 10 mmol/l Tris-HCl buffer pH 8.0 containing 1 mmol/l $MnCl_2$ and 0.02% NaN_3. This suspension was homogenized for 1 min at 22°C in a Virtis homogenizer and clarified by centrifugation. The supernatant was dialyzed overnight at 4°C with stirring against 900 ml of 1 mmol/l $MnCl_2$ containing 5 g charcoal.

Column chromatography

Human cerebral cortex (25 g) was homogenized at 22°C with 50 ml of 5 mmol/l Tris-HCl buffer pH 8.0. The homogenate was centrifuged and the pellet was extracted with 50 ml of the same buffer. The combined extracts (71 ml) were applied to a 2 × 44 cm bed of DEAE-cellulose which had been equilibrated with the Tris buffer. The column was washed with this buffer until the absorption of the eluate at 280 nm dropped below 0.05. An 800-ml linear NaCl gradient (0–0.5 mol/l) in the same buffer was then passed through the bed. The procedure was conducted at 22°C and the eluate was collected in 12.5-ml fractions.

The molecular weights of enzymes were estimated using the procedure of Whitaker [9]. A 1.5 × 112 cm bed of Sephadex G-200 was eluted with 10 mmol/l phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.02% NaN_3. Calibrating proteins were aldolase (M_r = 160 000), bovine serum albumin (M_r = 67 000), and ovalbumin (M_r = 45 000).

Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out according to Davis [10] using a stacking gel, but no sample gel. Tissue (2 g) was homogenized with 3 ml H_2O; the extract was dialyzed overnight at 4°C against 0.1 mmol/l $MnCl_2$. Extract (25 µl) was placed on the gel; electrophoresis and enzyme assay were performed as previously described [8] except that the assay was carried out under optimum conditions for tissue carnosinase.

Isoelectric focusing in a sucrose gradient was conducted according to Behnke et al [11]. A brain extract was prepared as for polyacrylamide gel electrophoresis; 2 ml were used in a 4-ml sucrose gradient containing 2.5% ampholyte, pH 3–10.

Results

Serum carnosinase assays

Serum samples from the homocarnosinosis patients and from healthy controls were analyzed for activity against carnosine and homocarnosine. As shown in Table I, the control samples were within the normal range, whereas the patient samples displayed little or no activity against carnosine or homocarnosine.

TABLE I

Activity of homocarnosinosis patient and control sera against carnosine and homocarnosine

Three different samples from each patient were analyzed with good agreement between replicates

Sample	Sex	age	μmol·ml^{-1}·h^{-1} vs.	
			carnosine	homocarnosine
Patient A	M	42	0.3	0.10
Patient B	M	38	0.1	0
Patient C	F	44	0	0
Control A	M	50	20	0.45
Control B	F	49	23	0.5
Control C	M	30	20	0.63
Adults ($n = 19$)	M and F	> 21	18–72	0.4–1.6

Optimum conditions for the hydrolysis of homocarnosine

Crude brain extracts, serum samples, and partially purified serum carnosinase [8] were all analyzed under various conditions so as to ascertain the optimum conditions for the hydrolysis of homocarnosine. The three preparations were indistinguishable in regard to the parameters investigated. Several buffers were compared, and Tris-HCl gave the highest activity. The optimum pH was 7.6. Many divalent metal ions were tested, and cobalt was the most effective activator. A K_m value of 0.4 mmol homocarnosine per l was obtained for all three preparations.

Anion exchange chromatography of human brain extracts

An extract of normal human cerebral cortex was chromatographed on a DEAE-cellulose column at pH 8.0 as described in the 'Methods' section. The effluent fractions were analyzed for their activity against carnosine and homocarnosine with the results shown in Fig. 1. Two peaks of activity were detected. Peak I was active against carnosine under tissue and serum carnosinase assay conditions, but was inactive against homocarnosine. Peak II fractions hydrolyzed carnosine and homocarnosine, being more active under serum carnosinase assay conditions than under the tissue carnosinase optimum conditions. No other peaks of activity against either of these substrates were detectable in the fractions before the salt gradient or the fractions eluted between 0.3 and 0.5 moles/l NaCl. The experiment shown in Fig. 1 was repeated four times with a similar outcome each time.

The peak I fractions were pooled, concentrated by ultrafiltration on a YM30 membrane, and chromatographed on a calibrated Sephadex G-200 column. The peak II fractions were handled similarly. The peak I and II enzymes had apparent molecular weights of 90000 and 160000, respectively, and only the latter was active against homocarnosine. Earlier results from this laboratory [8] showed that tissue carnosinase had a M_r of 90000 and did not hydrolyze homocarnosine, while serum carnosinase had a M_r of 160000 and hydrolyzed homocarnosine about 5% as fast as it split carnosine. The isoelectric points of brain and serum carnosinase were found to be approximately 5.6 and 4.7, respectively [8]. Therefore serum carnosinase would

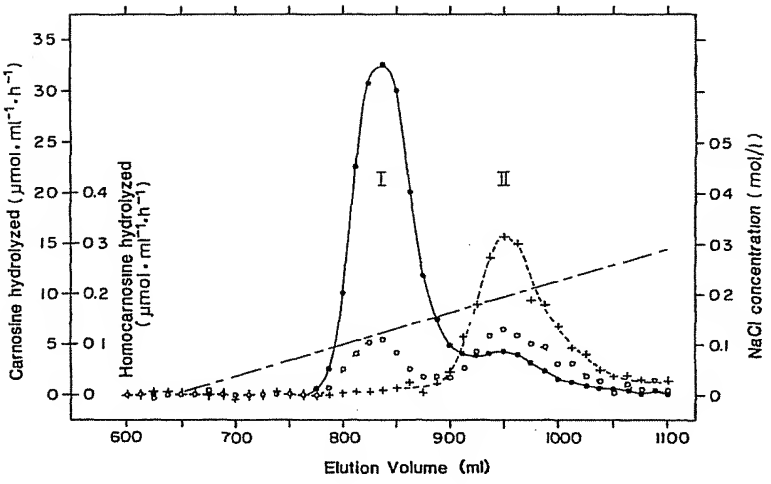

Fig. 1. DEAE-cellulose column chromatography of a crude human cerebral cortex extract. (●) activity vs. carnosine as measured by the tissue carnosinase assay; (○) activity vs. carnosine as measured by the serum carnosinase procedure; (+) activity vs. homocarnosine.

be expected to elute after brain carnosinase during DEAE-cellulose chromatography.

Tissue carnosinase (human kidney) and serum carnosinase were partially purified as described earlier [8]. When a mixture of these two preparations was chromatographed on DEAE-cellulose, the tissue carnosinase eluted early in the salt gradient and the serum carnosinase emerged thereafter.

It was concluded that peak I is brain carnosinase and peak II probably represents serum carnosinase, which was present because of the blood retained in the vessels of the brain sample. Furthermore, human brain probably does not contain the enzyme homocarnosinase, since the serum carnosinase fractions were the only peak of activity against homocarnosine which could be detected in the DEAE-cellulose column eluate. Peak II (M_r 160000) differs greatly from hog kidney homocarnosinase, which had a M_r of 57000 (4).

Analysis of human tissues for activity against homocarnosine

Fourteen human tissues were analyzed for their ability to hydrolyze homocarnosine. The tissues were brain, kidney, uterus, liver, lung, spleen, heart, skeletal muscle, stomach, small intestine, adrenal gland, pancreas, ovary, and testis. The activity ranged from 0.04 to 0.30 $\mu mol \cdot g^{-1} \cdot h^{-1}$. The activity of normal serum, which varies greatly from one person to another, ranged from 0.4 to 1.6 $\mu mol \cdot ml^{-1} \cdot h^{-1}$. Therefore, the tissues were approximately 10 to 20% as active as serum. If the tissues contained 10 to 20% blood, it would appear that their homocarnosine-splitting activity could be attributed to the serum carnosinase present in the trapped blood. This percentage of trapped blood agrees roughly with data in the literature [12]. In

view of this result, and the failure to detect homocarnosinase in brain, it seems likely that this enzyme may be absent from human tissues.

Serum carnosinase in CSF

Fifteen samples of human CSF were analyzed for carnosinase activity using the serum carnosinase assay procedure. Activity ranged from 1.2 to 10.0 μmol · ml^{-1} · h^{-1}; this compares with serum values which range from 18 to 72 μmol · ml^{-1} · h^{-1}. The average CSF sample was 10% as active as the average serum sample.

These CSF samples were also analyzed using the optimum conditions for tissue carnosinase; activity was only 4% of that measured using the serum carnosinase procedure. The CSF samples split homocarnosine approximately 3% as rapidly as they hydrolyzed carnosine using the serum carnosinase assay.

Pooled samples of CSF were concentrated 5-fold by ultrafiltration on a YM30 membrane. The retentate was placed on a calibrated Sephadex G-200 column; the carnosinase had exactly the same elution volume as serum carnosinase (M_r 160 000). Because of the molecular weight and the optimum conditions for activity, it was concluded that the CSF enzyme was probably serum carnosinase.

A sample of CSF from one of the homocarnosinosis patients (Patient A, Table I) was analyzed and found to be inactive against carnosine and homocarnosine. This observation is consonant with the notion that the CSF carnosinase is of serum origin.

Polyacrylamide disc gel electrophoresis

An extract of human kidney was subjected to polyacrylamide disc gel electrophoresis according to Davis [10]. As shown in Fig. 2, two carnosinase peaks were detected. The larger one ($R_F = 0.41$) undoubtedly represents tissue carnosinase, while the smaller one ($R_F = 0.61$) probably represents serum carnosinase. When serum was electrophoresed under the same conditions, the carnosinase R_F value

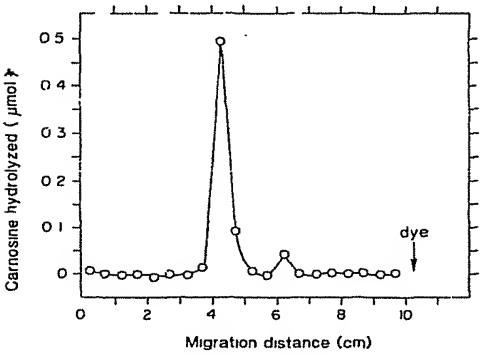

Fig. 2. Polyacrylamide disc gel electrophoresis of a crude human kidney extract at pH 8.9 in a 7.5% gel. After electrophoresis, 5-mm sections of the gel rod were macerated and analyzed for activity vs. carnosine under optimum conditions for tissue carnosinase.

averaged 0.58 [8]. When extracts of brain or placenta were subjected to electrophoresis, two peaks of carnosinase activity similar to those in Fig. 2 were observed. In all cases, the smaller peak ($R_F = 0.54–0.62$) migrated faster than the larger peak ($R_F = 0.38–0.45$). No activity against homocarnosine was detectable in the brain, kidney, or placenta gels. Although serum carnosinase hydrolyzes homocarnosine, the amount of this activity in the small quantity of extract (25 μl) applied to the gel was below the limit of detection.

Murphey and coworkers [13] found two major forms of carnosinase when extracts of human tissues were subjected to starch block electrophoresis. However, aside from the relatively small serum carnosinase 'contaminant', we have detected only one form of tissue carnosinase in extracts of human organs.

Isoelectric focusing

When a crude cerebral cortex extract was subjected to isoelectric focusing in a sucrose gradient, tissue carnosinase focused at pH 5.6. No serum carnosinase was detectable because this enzyme is unstable at its isoelectric point of 4.7 [8]. If homocarnosinase were present and stable at its isoelectric point, a peak of activity against homocarnosine should have been present; however, no activity against this substrate was detected. Hog kidney homocarnosinase was stable during focusing and had an isoelectric point of 5.6 [4].

Discussion

The serum carnosinase deficiency of the homocarnosinosis patients explains why they metabolized anserine and carnosine more slowly than normal controls [7]. Earlier, Perry and coworkers [14] had shown that carnosinemia patients were lacking serum carnosinase and metabolized carnosine and anserine at a lower rate than normal children.

We were unable to detect the enzyme homocarnosinase in normal human brain. None was detected when a brain extract was subjected to isoelectric focusing. When a brain extract was chromatographed on a DEAE-cellulose column, tissue and serum carnosinases were separated and all of the homocarnosine-splitting activity was present in the serum carnosinase peak. This enzyme was present because of the serum trapped in the blood vessels of the brain sample. Thirteen other human tissues were very low in homocarnosine-splitting activity and thus it is likely that their activity is also attributable to trapped serum carnosinase.

When Perry and coworkers [5,6] assayed human brain samples for homocarnosine-splitting activity, their assay parameters were very similar to the optimum conditions we developed for the hydrolysis of homocarnosine by serum carnosinase (pH, substrate concentration, and cobalt as the activating metal ion). They reported that the rate of homocarnosine hydrolysis in different regions of the brain ranged from 0.09 to 0.85 $\mu mol \cdot g^{-1} \cdot h^{-1}$ [5]. The activity of each region also varied from one individual to another. We found that the homocarnosine-splitting activity of serum ranged from 0.4 to 1.6 $\mu mol \cdot ml^{-1} \cdot h^{-1}$ in 18 adults (Table I). Therefore, the wide range of activity found in the brain may be explained by variation between

individuals in the concentration of carnosinase in serum, and variations between regions of the brain in the amount of trapped blood.

It has been observed that an unidentified enzyme in normal human CSF converts the homocarnosine in CSF to γ-aminobutyric acid and histidine [15]. Since serum carnosinase is present in CSF, this appears to be the enzyme responsible for the hydrolysis. The activity of this enzyme in CSF was weak [15], probably because of low concentrations of the necessary activating metal ions. The absence of serum carnosinase from the CSF of a homocarnosinosis patient explains the elevated concentrations of homocarnosine in the CSF of these patients. Similarly, carnosinemia patients, who also lack serum carnosinase, have elevated CSF concentrations of homocarnosine [16], although the symptoms of carnosinemia are different from those of homocarnosinosis.

Although we did not detect homocarnosinase in human tissues, it is present in hog kidney and in certain rat tissues [4]. Homocarnosinase must be a cellular enzyme in these animals because carnosinase was not detectable in rat or hog serum [4,8].

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